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Development of a Solid-Supported Glaser-Hay Reaction and Utilization in Conjunction with Unnatural Amino Acids

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COMPLIANCE PAGE

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ABSTRACT

Polyynes, or acetylenic scaffolds, are often found as core structures in various natural products and exhibit biological properties such as antibacterial, antifungal, and anti-cancerous activity. This research develops a new methodology, a solid-supported Glaser-Hay reaction, alleviating the standard chemoselectivity issues associated with the Glaser-Hay reaction and providing a means to synthesize these polyyne structures in a chemoselective fashion. To extend the biological benefits of the solid-supported Glaser-Hay reaction, this methodology is then employed toward the synthesis of natural products. Furthermore, utilizing unnatural amino acid technology, this reaction can be transferred to a biological context, serving as a novel bioconjugation method and providing a new methodology for potential therapeutic applications.

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Finally, special thanks to my family and to ADR, who supported me every single step of the way, brought me up when I was down, and loved me unconditionally through it all.

For Grandma,
This road would have been a heck of a lot harder without you.

Chapter 1- Introduction to Unnatural Amino Acids

I. Expanding the Genetic Code

Amino acids possess both a carboxylic acid and an amino functionality that facilitate their polymerization to form peptide bonds. Consequently, these functional molecules comprise the fundamental building blocks of proteins. Overall, amino acids play an important role in determining protein function, as well as in cell signaling, gene expression, and hormone synthesis.[1] With 20 canonical amino acids, the potential number of proteins afforded by their combination in different number and sequence provides a wide array of protein structures and functions. However, it would be beneficial to add new amino acids to this makeup, allowing for varied or improved post-translational protein function, new therapeutic methods, insights into numerous biological processes, and the production of new proteins or organisms.[2] Therefore, a substantial amount of research has been conducted to introduce unnatural amino acids (UAAs) in a specific fashion into the sequence of a protein. UAAs allow for the expansion of the genetic code, affording routes for further control of known protein functions as well as a methodology for generating new proteins with enhanced structure and function. To date, hundreds of amino acid derivatives have been synthesized to include functionalities such as metal binders, azides, fluorophores, photolabile groups, and alkynes.[2] These functional groups allow for a vast array of new probes for characterization, bioconjugation methods, and

medicinal and therapeutic studies. Overall, the utilization of unnatural amino acids allows for novel protein research.

II. Protein Translation

Translation is an intricate biological process that involves three main steps: initiation, termination, and elongation. During initiation, the functional ribosome is assembled in response to a special initiation codon in the mRNA. Next, during elongation, aminoacyl-tRNAs (aa-tRNA) enter the process and are used as delivery devices. The aa-tRNA binds an amino acid ("charges" the amino acid) and delivers the AA to the ribosome for incorporation into the growing polypeptide chain in response to a specific codon. In order to function correctly, the aminoacyl-tRNA must be properly charged with an amino acid as well as contain the proper codon for recognition of the mRNA site. The ribosome then catalyzes the formation of a growing polypeptide chain which continues to elongate with each sequential codon. Finally, during termination, a stop codon is reached, and the newly formed polypeptide chain is released from the ribosome.[3] (Figure 1.1)

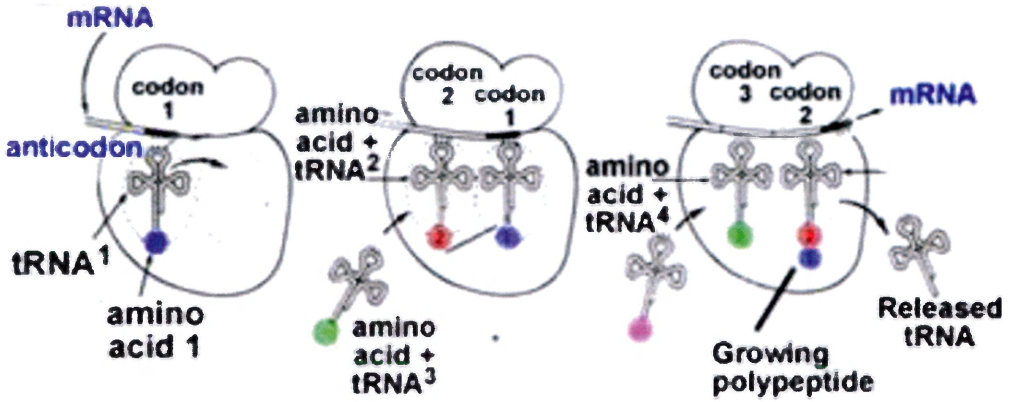

Figure 1.1: Each tRNA is charged with the appropriate amino acid, then responds to a specific anticodon located on the mRNA template. The polypeptide chain continues growing until a stop codon is reached on the mRNA. [4]

In order for this process to be successful, it is necessary that the tRNA is properly charged with the correct amino acid, as each tRNA harbors a specific anticodon that allows for the translation of the nucleic acid message into a protein context. This process is done by an aminoacyl tRNA synthetase (aaRS) that loads a specific amino acid onto an isoacceptor tRNA. Each amino acid requires a unique aaRS for charging its isoacceptor tRNA that is said to be orthogonal to the other aaRSs. Once properly loaded, the aminoacyl-tRNA can then base-pair with the proper codon located on the mRNA.[5] Intricate translational machinery then is employed in order to form peptide bonds between the growing chain of amino acids, and the process is terminated when the stop codon located in the mRNA is reached.

III. Manipulating Translational Machinery to Incorporate UAA's

There have been many methodologies proposed for incorporation of UAAs *in vivo*, with one of the most accepted and successful approaches being established by the Schultz laboratory. Overall, this method manipulates the natural translational machinery of proteins in order to site-specifically incorporate the desired UAA.

As previously described, each amino acid is charged onto a specific aminoacyl-tRNA that responds to its codon on the mRNA in order to insert an amino acid and generate a polypeptide chain. The Schultz method exploits this machinery in order to incorporate an unnatural amino acid. To begin, the UAA must be charged specifically onto the aminoacyl-tRNA. It is necessary for this tRNA to not be recognized by any native aminoacyl-tRNA synthetases in order to prevent the loading of native amino acids to the tRNA. Also required is an aminoacyl-tRNA synthetase (aaRS) that will not recognize native aminoacyl-tRNAs. This independence from the endogenous system is often referred to as being "orthogonal" to the other aaRSs and tRNAs. An efficient method for generating an orthogonal aaRS/tRNA pair for use in *E. coli* is importing an already existing pair from a eukaryote or archaeon.[6] In doing so, the new aaRS will not charge native amino acids onto the tRNA of the *E. coli*, and similarly, the native *E. coli* aaRSs will not recognize the foreign tRNA. To date, several of these orthogonal aaRS/tRNA pairs have undergone a selection process and have been adapted for use in *E. coli*.[6]

The selection process was initially performed using an aaRS/tRNA pair from the archaea *Methanocaldococcus jannaschii*, *Mj*TyrRS/*Mj*tRNA^{Tyr}.[9] In order to alter the aaRS,

(here the *Mj*TyrRS) to specifically recognize a desired unnatural amino acid, a large library of the mutant *Mj*TyrRS was prepared and underwent a two-step selection process involving both a positive and a negative selection step. (Figure 1.2)

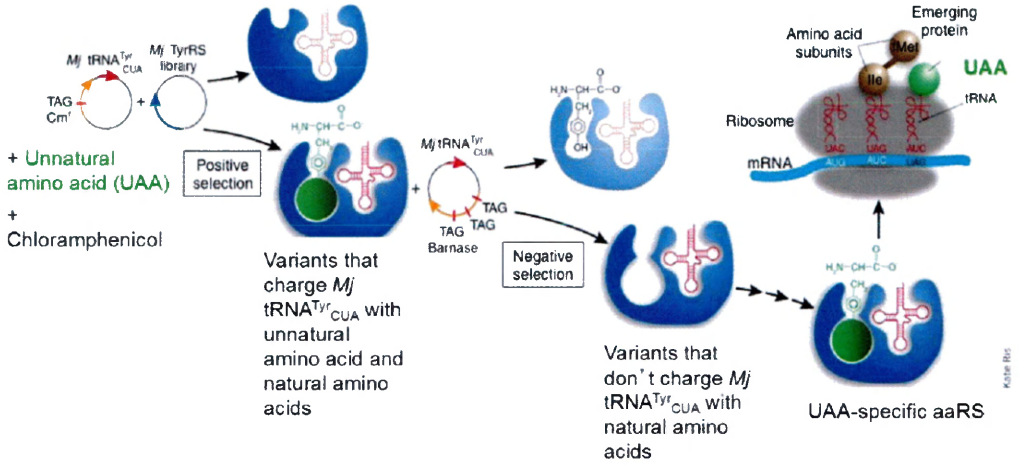

Figure 1.2: Demonstration of the selection process to produce a mutant *Mj*TyrRS/*Mj*tRNA pair that will successfully incorporate a specific unnatural amino acid. This process is completed 2.5 times, ultimately ending on a positive selection step.

In step one of the selection process, the mutant cells transformed with an aaRS/tRNA pair are grown in the presence of chloramphenicol and the desired unnatural amino acid to assess which mutants have the ability to suppress the amber stop codon (TAG) and successfully insert an amino acid. If the TAG codon is suppressed and the amino acid inserted, the cells will carry the gene for resistance to choloramphenicol (chlor) and therefore will survive. Any cells not surviving did not possess the ability to successfully suppress the TAG codon and will die in the presence of chlor. Therefore, survivors of this

step are mutant *Mj*TyrRS that can successfully aminoacylate *Mj*tRNA with the desired UAA or an endogenous amino acid. These survivors then undergo a negative selection step to further provide selective *Mj*TyrRS mutants, eliminating those that accept an endogenous amino acid. In this step, the mutant aaRS/tRNA are grown without the unnatural amino acid, and any pairs that suppress the inserted TAG codons of the barnase gene and insert an endogenous amino acid are eliminated due to the production and toxicity of barnase.[9] Survivors of this step then undergo the entire process again and ultimately end on a positive selection step, ultimately producing a very selective *Mj*TyrRS/*Mj*tRNA^{Tyr} pair for unnatural amino acid incorporation. If the selection process is not completed correctly or enough rounds are not performed, the aaRS/tRNA pair may not be completely selective and is referred to as 'promiscuous,' meaning that this synthetase has the ability to charge numerous amino acids to the tRNA.

Moving forward, in order for the orthogonal aaRS/tRNA machinery to be effective *in vivo*, there must be a codon located on the mRNA that recognizes the anticodon for the orthogonal tRNA charged with the UAA. This provides site-specific incorporation of the UAA based on the mRNA sequence. The degeneracy in the genetic code proves extremely beneficial in this step of the methodology. Because there are four nucleotides and three nucleotides make up any given codon, there are a possible sixty four codons (4^3); sixty one of these codons are associated with one of the twenty amino acids, and three are recognized as stop codons. Fortuitously, the TAG codon (amber stop codon) is a degenerate codon that denotes termination and can be exploited in order to site-specifically incorporate unnatural amino acids.[5] This is achieved by mutating the DNA that

produces the mRNA to include the TAG codon. The tRNA charged with the UAA then is able to complimentarily base pair to the TAG region, allowing transcriptional machinery to continue the process, and incorporating the UAA site-specifically. (Figure 1.3) If there is no amino acid present, translation is terminated in response to the TAG stop codon.

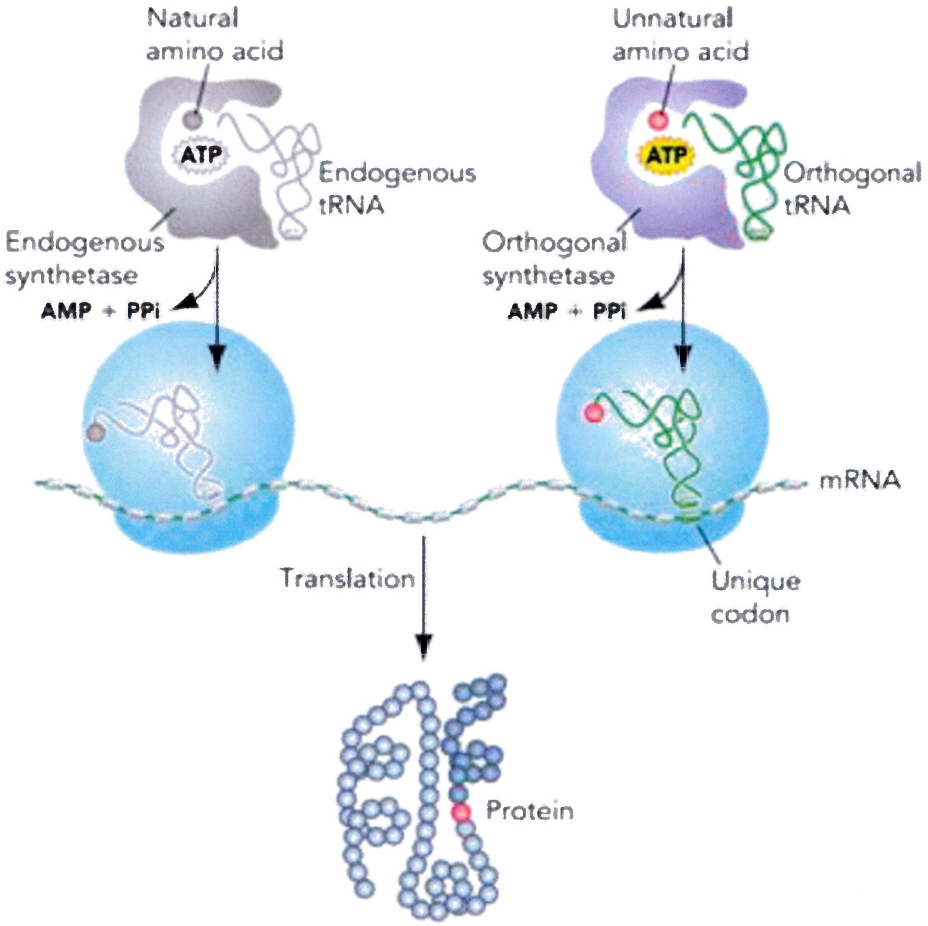

Figure 1.3: Normal translation can be seen on the left, where a tRNA synthetase charges a specific amino acid to the tRNA, which then base pairs with its anticodon in the mRNA template and inserts the amino acid into the expanding polypeptide chain. On the right, an introduced orthogonal tRNA synthetase charges the orthogonal tRNA with the

unnatural amino acid, and specifically responds to a mutated codon in order to base pair with the mRNA and allow translation to continue, site-specifically incorporating the UAA.[7]

In order to utilize this developed technology, *E. coli* must be transformed with two separate plasmids, one encoding for the orthogonal aaRS/tRNA pair, as well as one encoding for the mutant protein of choice containing the TAG codon. As mentioned, it is possible for the orthogonal synthetase to be 'promiscuous.' This degree of promiscuity can, at times, prove beneficial. When attempting to incorporate a newly described unnatural amino acid, the researcher may run a screen of created synthetase/tRNA pairs in hopes that an already evolved pair will charge their UAA appropriately. This eliminates the need for a tedious selection process for every single created UAA, and may provide a quick route for the incorporation of numerous UAAs.[8] Once transformed with both the orthogonal aaRS/tRNA and mutant protein, cells containing these plasmids can be grown on a plate containing antibiotics associated with both plasmids, and only cells that have successfully been transformed with both are able to survive. Colonies can then be chosen for expression with the desired UAA. Once the UAA is introduced, it will be site-specifically incorporated at the mutated TAG site of the protein, or conversely, when not expressed with the UAA, translation of the protein will be terminated by the amber stop codon. This feature allows for a viable control method determining if the desired UAA was site-specifically incorporated. Overall, this methodology is a useful mechanism for the specific introduction of UAA's *in vivo*, expanding the genetic code and allowing for new and exciting research on protein structure and function

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Chapter 2- Incorporation of a Terphenyl Unnatural Amino Acid

I. Introduction

A. Fluorescent Probes as Protein Markers

Introducing fluorescent probes into proteins is a useful tool to examine protein structure and function, as well as for facilitating visualization of cellular location of a given protein.[1] To date, there are numerous known fluorescent probes that can be used to further our scientific understanding of different proteins and their cellular functions. These include small organic fluorescent dyes, nanocrystals (quantum dots), naturally occurring fluorescent proteins, and small genetically encoded tags that can be complexed with fluorophores.[2] These probes may also be used in different combinations, allowing for a wide array of fluorescence studies in the fields of molecular biology, organic and analytical chemistry, and materials science.[2] Different fluorophores possess unique spectral properties, and can therefore be multiplexed towards a wide array of protein applications. This necessitates the need for a plethora of known fluorophores with unique properties, as well as continued research to expand the number and physical/chemical properties of these fluorescent protein probes.[3-5]

There are varying mechanisms reported in literature that may be employed to introduce fluorescent probes into proteins. One noted method introduces a fluorophore to a protein post-translation via chemical modification.[6] However, this methodology is limited to the number of surface-exposed, reactive residues on the protein that are

available for attachment of the probe. Other limitations to this approach involve issues associated with non-specific labeling and a general lack of control over the sites that react with the fluorophore leading to complex mixtures both in residues and number of conjugated probes.[6]

Another reported methodology uses chemical modification of unique unnatural amino acid residues to afford a degree of specificity to the probe conjugation. Early studies employed chemically misacylated tRNA to introduce an unnatural amino acid. This amino acid residue could be the fluorophore itself, or could be further used to attach the desired probe. Using a misacylated tRNA approach to incorporate a UAA, however, affords limited protein yields and can only be performed on easily accessible positions on the protein.[7,8,9] To avoid these standard limitations, an unnatural amino acid can be incorporated *in vivo* through the use of an amber suppression system developed in the Schultz laboratory.[19] (See Chapter 1) Specifically, we aim to introduce a terphenyl UAA fluorescent probe into proteins (Figure 2.1) via this site-specific unnatural amino acid methodology. Moreover, we will investigate the role of the terphenyl UAA on the alteration of GFP fluorescence to illustrate its unique fluorescent properties.

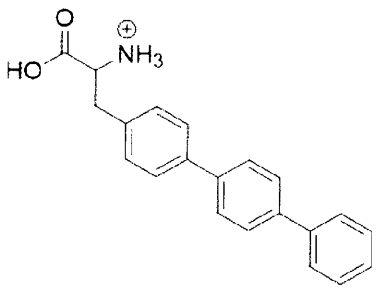


Figure 2.1: Structure of terphenyl (4-biphenyl-L-phenylalanine)(**4**).

The proposed methodology utilizing an orthogonal tRNA and aminoacyl-tRNA synthetase (aaRS) to incorporate an UAA via response to an introduced TAG codon has long been used as a successful approach for incorporation of UAA's.[10] Ideally, we hope to exploit the recently discovered promiscuity of aaRSs in order to obviate the need for an intensive aaRS evolution. We hypothesize that a promiscuous aaRS can effect site-specific incorporation of the terphenyl UAA to produce a novel terphenyl enhanced GFP.[11]

B. Benefits of Terphenyl as a UAA

Due to the extensive π-conjugation within the structure of the terphenyl, this compound proves to be an ideal and interesting compound to use as a fluorescent probe in proteins. As a fluorophore, terphenyl displays photophysical properties and has been characterized and studied by various research groups. This molecule displays an emission wavelength of 342 nm and has been discovered to be sensitive to environmental conditions.[12] Due to these properties, terphenyl has found use in multiple applications such as two-photon laser scanning microscopy,[13] femtosecond fluorescence spectroscopy,[14] and α-helical secondary structure investigations.[15] By using these various methods of study, terphenyl has proven to be of use in expanding knowledge of charge transfer, isomerization, and protein dynamics within biological systems.

Furthermore, terphenyl UAA incorporation proves to be a useful mechanism for fluorescence labeling of a desired protein. The terphenyl UAA, although containing a

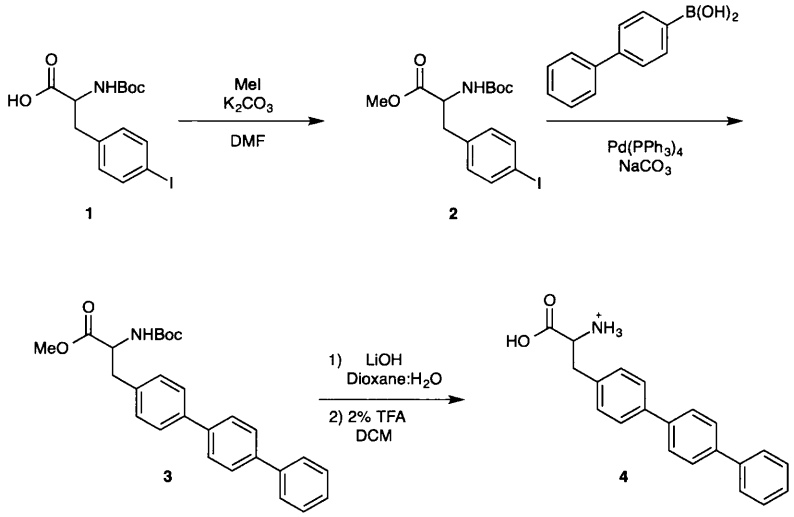
triphenyl moiety, is of relatively small size which allows for conformational mobility within the protein. Benefits of using terphenyl as a fluorescent UAA have recently been examined by Chen and co-workers.[16,17] This group incorporated terphenyl and its derivatives into dihydrofolate reductase (DHFR) from *Escherichia coli* for FRET fluorescence studies. Their studies report the successful incorporation of terphenyl and an increased knowledge of changes in protein conformations and dynamics. However, the method of incorporation of the UAA involved chemically misacylated tRNAs. As previously discussed, this method can result in lower protein yields and requires tedious synthetic modifications of tRNAs. Due to the demonstrated beneficial application of terphenyl as an UAA, we set forth to incorporate terphenyl via the orthogonal tRNA and aminoacyl-tRNA synthetase system in order to completely incorporate it into the genetic code site-specifically, and study the unique effects this molecule displays in GFP.

II. Results and Discussion

A. Synthesis of a Terphenyl UAA

Studies were initiated with the synthesis of the terphenyl UAA from an adapted literature protocol.[17] An initial esterification of N-Boc-4-iodo-L-phenylalanine (**1**) with methyl iodide affords the protected methyl ester (**2**, 90% yield). A Suzuki cross coupling reaction was then employed with 4-biphenylboronic acid to yield the protected terphenyl unnatural amino acid (**3**). Lastly, the Boc and methyl protecting groups were removed via LiOH and TFA to afford the fully deprotected 4-biphenyl-L-phenylalanine (**4**) (Scheme 2.1).

Scheme 2.1



Upon successful synthesis, the focus then shifted to incorporating this fluorescent UAA into the protein of choice. To date there are numerous synthetases that have been evolved to accept a wide array of UAAs.[18,19] Therefore, we attempted to identify an aaRS that would recognize the terphenyl UAA and charge the corresponding orthogonal tRNA in order to incorporate the fluorescent probe into GFP.

B. Incorporation of Terphenyl UAA into GFP

Green Fluorescent Protein (GFP) is a natural fluorescing protein that is originally isolated from a species of deep sea jellyfish, *Aequorea victoria*. GFP contains a natural, internal fluorophore which is located on an α-helix located in the inner protein structure. The fluorophore is surrounded by the outer β-barrels of the protein, that overall form a

cylindrical protein structure.[22] Due to the extensive fluorescent properties and characteristics of GFP, it is often used as a reporter gene, cell marker, or a fusion tag for biochemical applications.[22] Conveniently, our lab has access to GFP that has been mutated with TAG codons at the amino acid positions of 3, 66, 133, and 151. In this research, we chose to use these various GFP mutants for the incorporation of terphenyl.

In hopes of using an already evolved synthetase and avoiding a lengthy and tedious aaRS selection process, previously evolved synthetases were screened for use of incorporation of terphenyl into GFP. The synthetases initially screened were chosen based on their known 'promiscuity' (See Chapter 1) or their ability to incorporate UAAs that are structurally similar to the terphenyl moiety.

Incorporation studies involved the co-transformation of BL21(DE3) *E. coli* cells with a pET-GFP-TAG-151 plasmid and a plasmid encoding a known synthetase. A single colony was then selected and grown for expression in the presence of 100 mM **4**, or in the absence of the amino acid as a control. Following expression, the fluorescence of the GFP was measured using a plate reader (Figure 2.2).

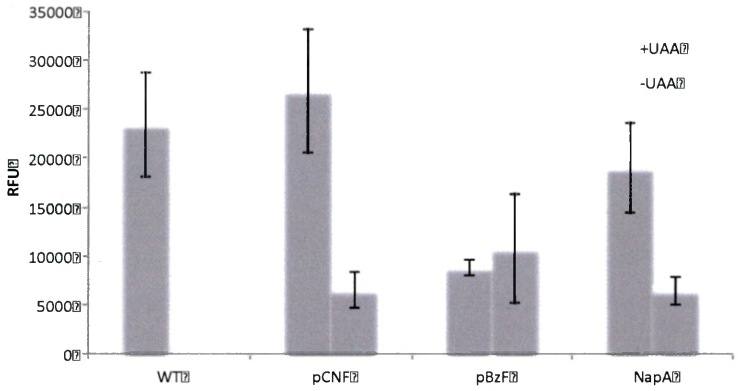


Figure 2.2: Plate reader data of initial screened synthetases pCNF, pBzF, and NapA. Tests were performed to measure fluorescence of purified protein with and without the incorporated UAA. The presence of fluorescence from the GFP shows that the protein is functional, therefore indicating successful incorporation of terphenyl.

As seen in Figure 2.2, cultures containing the pCNF and NapA aaRS showed differential fluorescence activity between the + UAA and –UAA expressions. The pCNF and NapA synthetases were evolved to specify a unique structural group. (Figure 2.3)

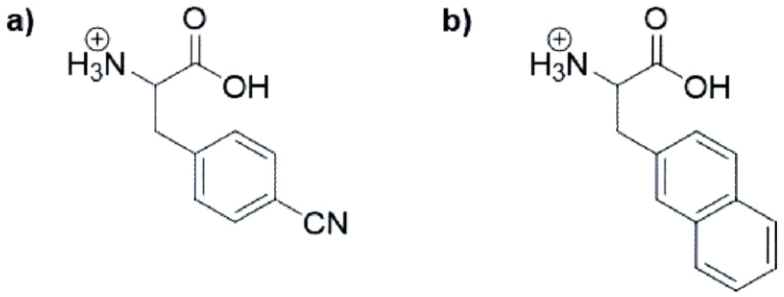


Figure 2.3: a) The pCNF synthetase was evolved to recognize a phenylalanine amino acid containing a cyano moiety, but displays a level of promiscuity for numerous other UAAs. b) NapA was evolved to recognize an alanine containing a naphthalene functionality. The structural similarity between this structure and the terphenyl plays a key role in the incorporation of the terphenyl into the protein.

Research has shown that the pCNF synthetase system displays a degree of promiscuity and is capable of recognizing multiple UAAs,[21] making this an ideal synthetase for use with terphenyl. The NapA synthetase was evolved to recognize naphthalene structures, which are structurally similar to terphenyl, also making this system ideal for use with terphenyl. To further confirm the incorporation of terphenyl, the GFP was purified using a His- Ni-NTA spin column and analyzed by gel electrophoresis. (Figure 2.4)

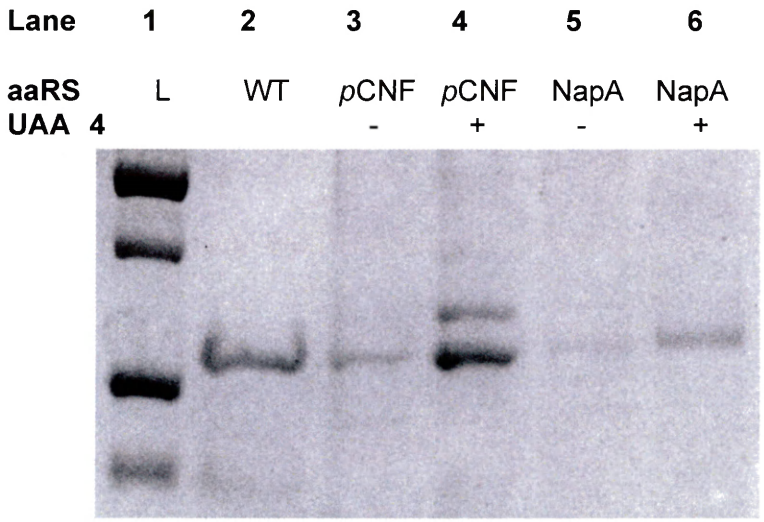


Figure 2.4: SDS-PAGE of GFP expression with cultures containing the terphenyl UAA. Lanes 3 and 4 confirm the presence of terphenyl using the pCNF aaRS due to the strong band presence in lane 4 and lack of, or weak presence, in lane 3. Similarly, the band in lane 6 indicates protein presence when using the NapA aaRS and expressing with terphenyl.

Gratifyingly, the incorporation of terphenyl was confirmed in both the pCNF and NapA synthetases. This incorporation was performed with the GFP-TAG-151 plasmid;

however, it would be advantageous to incorporate the probe at multiple residues to investigate the effect of different chemical environments on fluorescent properties. Thus, we set forth to incorporate **4** at different positions on GFP to validate its utility.

C. Using GFP Mutants for Fluorescence Studies

As previously mentiones, GFP plasmids containing the TAG codon at numerous sites within GFP have already been engineered. These positions include 3, 66, 133, and 151. Incorporating **4** at these different positions allows for further research of the effects of this UAA based on its structural location within the protein. The residues used for site-specific incorporation can be seen in Figure 2.5.

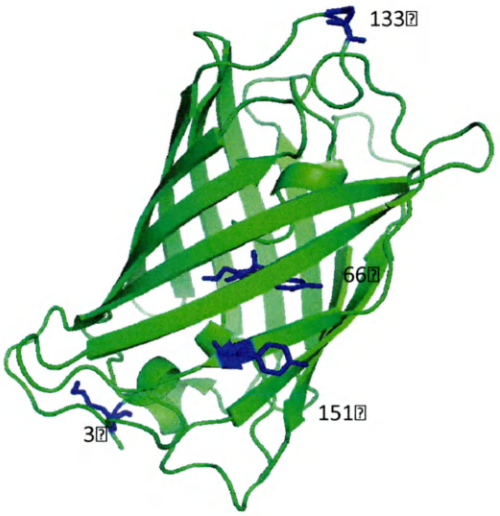


Figure 2.5: Crystal structure of GFP showing the different sites available for site-specific incorporation of terphenyl. Significantly, position 66 is located within the protein at the site of GFP's natural chromophore. Positions 3, 151, and 133 are located on the outer surface of the protein.

Each of these residues has a unique location in the protein that is beneficial when studying effects of an incorporated fluorescent probe. Position 66 is found on the inside of the protein, and is a key residue in GFP's natural fluorophore. Residue 3 is found at the N-terminus as part of a non-structured loop, and residue 133 is found opposite residue 3 in a segment harboring a structured loop. Position 151 is found on the end of a β-sheet that comprises the β-barrel of the protein, and is therefore the residue that is most rigid. By incorporating **4** at each of these residues and analyzing the fluorescence via excitation at 395 nm, dramatic effects can be seen that offer insight regarding the overall effects of this UAA on GFP. (Figure 2.6)

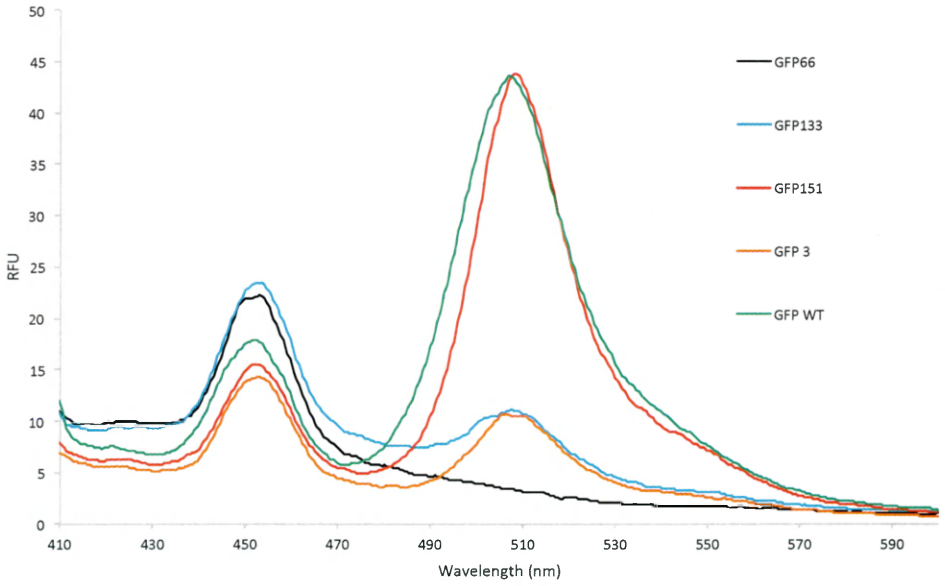


Figure 2.6: Emission spectra of GFP mutants containing **4** at each described residue. Protein was excited at 395 nm.

Overall, compared to the WT GFP spectrum (in green), each mutant containing the terphenyl displays a slight red shift to a higher wavelength. The 455 nm and 512 nm emissions seen in the WT protein, respectively, correspond to the protonated and deprotonated forms of tyrosine residue 66 located within the core fluorophore, and can be seen to be impacted significantly in some of these mutants. When terphenyl is inserted at residue 66, the 512 nm emission completely disappears in favor of the 455 nm emission, which is logical due to the loss of the hydroxyl group and its acid/base properties within the fluorophore. Inserted at residue 151, the terphenyl seems to have no effect on either the 512 nm or 455 nm emissions relative to WT, as position 151 is more rigid than the others, and therefore is not significantly altered due to this alteration. When the terphenyl is located at the more flexible positions 3 and 133, the 455 nm emission is more favored than the 512 nm emission, opposite of what is typically favored in WT. This indicates that inserting terphenyl at these positions causes an overall structural change in the protein, as well as change of the resting protonation state of the tyrosine located at position 66.

Furthermore, by exciting each mutant at 300 nm, we can also detect the terphenyl fluorescence within the GFP. (Figure 2.7)

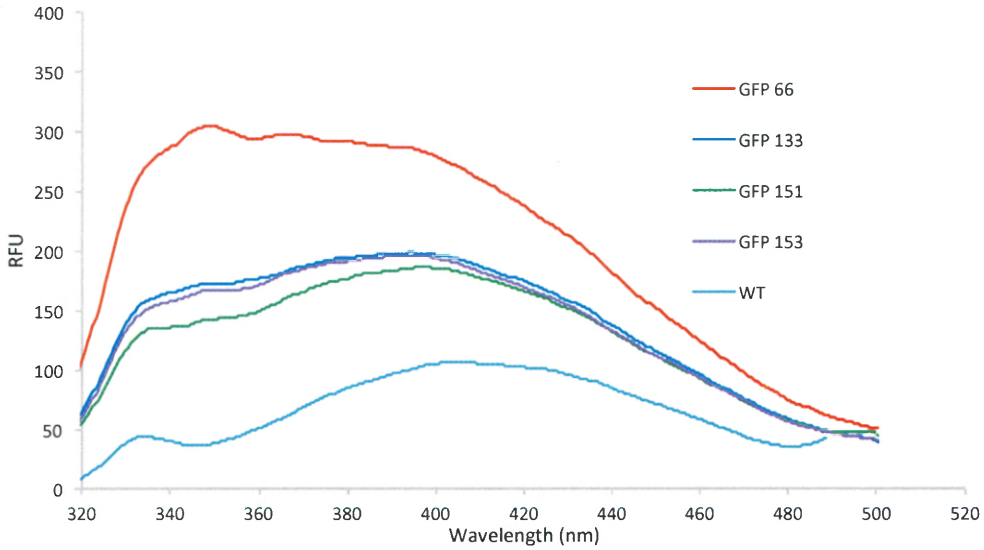


Figure 2.7: Fluorescence emissions spectra of each mutant GFP. Protein was excited at 300 nm.

According to literature, terphenyl displays an emission maximum at ~342 nm when excited at 300 nm.[12] As shown in Figure 2.7, WT GFP has only slight natural emission when excited at 300 nm. However, overall we can see that terphenyl alters the λ_{max} of the emission when inserted at each position. The new emission is shown to be broader and more intense. Specifically, when inserted at position 66, the emission shifts significantly, becomes much broader, and is double the intensity of the original WT.

Overall, by exciting the protein at both 300 nm and 395 nm, we are able to view altered fluorescence behavior of GFP due to the site-specific insertion of terphenyl. Hence, insertion provides a highly selective mechanism for tuning GFP fluorescence.

In conclusion, this research was able to incorporate a terphenyl moiety into GFP via use of an orthogonal tRNA and aminoacyl-tRNA synthetase system. This afforded

complete genetic and site-specific incorporation of the terphenyl UAA, which had not been previously accomplished. Furthermore, upon UAA incorporation, this research documented that terphenyl was an environmentally sensitive fluorescent probe in GFP, allowing for a better understanding of photo-physical behavior of GFP upon site-specific alteration.

III. Materials and Methods

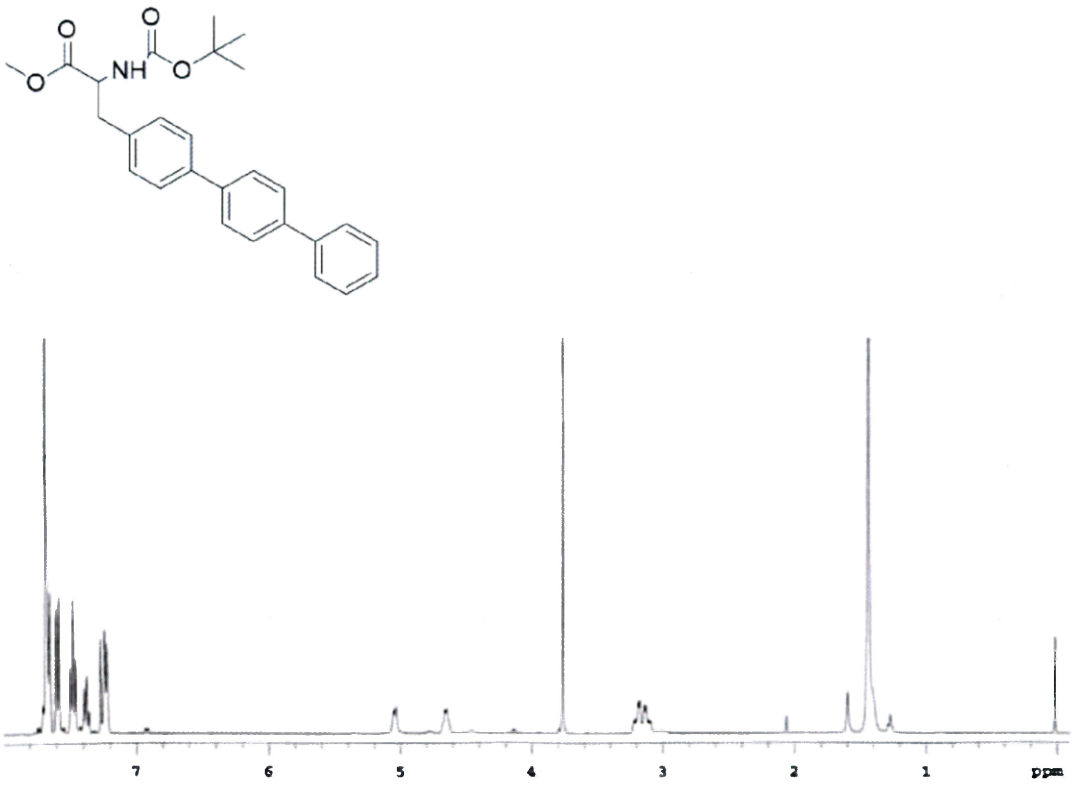
General. Solvents and reagents were obtained from either Sigma-Aldrich or Fisher Scientific and used without further purification, unless noted. Reactions were conducted under ambient atmosphere with non-distilled solvents. NMR data was acquired on a Varian Gemini 400 MHz. Fluorescence data was measured using a PerkinElmer LS 55 Luminescence Spectrometer. All GFP proteins were purified according to manufacturer's protocols using a Qiagen Ni-NTA Quik Spin Kit.

Synthesis:

2. N-(*tert*-butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester. To a solution of Boc-Phe(4-I)-OH (500 mg, 1.3 mmol) in 10 mL DMF was slowly added $NaHCO_3$ (323 mg, 3.8 mmol, 3 equiv.). Methyl iodide (97 uL, 1.9 mmol, 1.5 equiv.) was then added to the mixture and the reaction was allowed to stir under argon for 24 hours at 60 °C. The cooled reaction mixture was then extracted and washed with water and EtOAc (3 x 20 mL). The organic layer was dried with $MgSO_4$ and the solvent was removed *in vacuo* to leave a yellow oily solid. This product was then purified via silica gel chromatography with a gradient elution hexanes:EtOAc (3:1 → 1:1). N-(*tert*-butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester was obtained as a white solid (466 mg, 90%).

4. 4-biphenyl-L-phenylalanine. To a mixture containing N-(*tert*-butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester (200 mg, 0.24 mmol) and 4-biphenylboronic acid (202 mg, 1.02 mmol) in 1:1 THF:Toluene (24 mL) was added a solution of Na_2CO_3 (108 mg, 1.02 mmol) in water (10 mL). The mixture was degassed with a stream of argon and $Pd(PPh_3)_2Cl_2$ (15 mg, 0.02 mmol) was added slowly to the reaction. The reaction was then stirred vigorously at 80 °C for 16 hours. The cooled reaction was then extracted using EtOAc and water (3 x 20 mL each) and the organic layer was dried with $MgSO_4$. Solvent was removed *in vacuo*. The crude product was then purified on a silica gel column using hexanes:EtOAc (5:1→1:1). To remove the protecting group, a 1:1 LiOH/dioxane solution (2 mL) was added to the product on ice and stirred for 2 hours at room temperature. The dioxane was removed *in vacuo*. The aqueous solution was cooled on ice and 6 M HCl was added until a pH of 4 was achieved. The solution was extracted and washed with water and EtOAc and the organic layer was dried with $MgSO_4$, and then concentrated *in vacuo*. The yellow oil was resuspended in 50% TFA solution (500 µL TFA/500 µL DCM) on ice and allowed to stir at room temperature for 1 hour. The solvent was then removed *in vacuo* and product was obtained as a white solid (208 mg, 99%). ^{1}H NMR (400 MHz;CD_3OD): δ 7.20-7.75 (m, 18H), 4.20 (t, J=1.5 Hz, 1H), 3.05-3.35 (m, 2H)

^{1}H NMR of protected form of terphenyl:



^{1}H NMR (400 MHz; d-MeOH): δ 7.20-7.75 (m, 18H), 5.05-4.85 (m, 1H), 3.78 (s, 3H), 3.05-3.35 (m, 2H), 1.42 (s, 9H).

General GFP Expression. A pET-GFP-TAG variant plasmid (0.5 µL) was co-transformed with a pEVOL-*p*CNF plasmid (0.5 µL) into *Escherichia coli* BL21(DE3) cells using an Eppendorf eporator electorporator. The cells were then plated and grown on LB agar in the presence of chloramphenicol (34 mg/mL) and ampicillin (50 mg/mL) at 37 °C overnight. One colony was then used to inoculate LB media (4 mL) containing both ampicillin and chloramphenicol. The culture was incubated at 37 °C overnight and used to inoculate an expression culture (10 mL LB media, 50 mg/mL Amp, 34 mg/mL Chlor) at an OD_{600} 0.1. The cultures were incubated at 37 °C to an OD_{600} between 0.6 and 0.8 at

600 nm, and protein expression was induced by addition of the UAA (100 μL, 100 mM) and 20 % arabinose (10 μL) and 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; 10 μL). The cultures were allowed to shake at 30 °C for 16-20 h then centrifuged at 5,000 rpm for 10 minutes and stored at -80 °C for 3 hours. The cell pellet was re-suspended using 500 μL of Bugbuster (Novagen) containing lysozyme, and incubated at 37 °C for 20 minutes. The solution was transferred to an Eppendorf tube and centrifuged at 15,000 rpm for 10 minutes, then the supernatant was poured into an equilibrated His- pur Ni-NTA spin (Qiagen) column of nickel resin (200 μL) and GFP was purified according to manufacturer's protocol. Purified GFP was analyzed by SDS-PAGE (BioRad 10% precast gels, 150V, 1.5h), and employed without further purification.

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Chapter 3- Toward Incorporation of a Photocaged Selenocysteine

I. Introduction

A. Selenocysteine as the 21st Amino Acid

Traditionally speaking, there are 20 known amino acids; however, the selenium containing amino acid Selenocysteine (Sec, U) is often referred to as the 21st.[4] (Figure 3.1) Research on this unique amino acid has picked up in the last decade due to findings that suggest it may be employed in cancer prevention.[1] Selenium derivatives have been used as successful tumor-suppressant agents in rodents[2] and have the potential ability to act as chemoprotectors against negative side effects of drugs.[3] Moreover, at least 25 selenoproteins are known in humans, including glutathione peroxidase and thioredoxin reducatase. Selenoproteins exhibit important functions in humans, such as control of our cells oxidation state, sperm maturation, brain development, and endocrine system function.[9] Despite all of the current knowledge surrounding this amino acid, many biological roles of selenocysteine remain undiscovered. Consequently, due to the significant biological relevance of this amino acid, continued research of selenocysteine and its derivatives are of high importance in the fields of biochemistry and medicinal chemistry.

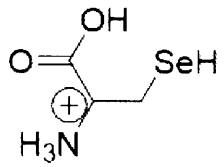


Figure 3.1: Structure of Selenocysteine.

Selenocysteine exhibits the three main characteristics common to the twenty canonical amino acids: it is inserted during translation of mRNA, has its own codon (UGA), and has its own unique tRNA.[5] The UGA codon that recognizes Sec, however, also naturally serves as a stop codon. In order to encode the UGA codon to act as a recognition element for selenocysteine, a unique structure in an untranslated portion of the mRNA called a selenocysteine insertion sequence (SECIS) is required. Unfortunately, this machinery varies in prokaryotic cells and eukaryotic cells, making incorporation and recognition of selenocysteine a difficult task.[6] One method for overcoming this barrier is using solid phase peptide synthesis in combination with native chemical ligation. Early selenocysteine research often involved the use of an Fmoc and Mob group as the amino acid protection functionality (forming a Fmoc-Sec(Mob)-OH protected AA). This provided several disadvantages, however. The use of Mob often caused racemization, and deprotection of the Fmoc group included the use of piperdine-which was shown to cause deselenation of the amino acid, forming an alanine. To alleviate this, focus of selenocysteine protection shifted to the use of Boc/Benzyl groups for protection.[5] Using the Boc group allows for pH controlled deprotection, utilizing a more acidic/neutral environment for deprotection and cleavage protocols.[8] A benzyl functionality is used

alongside of a Boc group as a protection group for the selenium ion. Another reported method to incorporate selenocysteine into proteins involves chemically converting serine to selenocysteine; however, these conversions are often cumbersome and do not achieve site-specificity.[10]

To alleviate these issues, we propose incorporating a selenocysteine UAA into proteins using site-specific genetic insertion. This has been attempted previously, but the inherent reactivity of selenocysteine has prevented successful evolution of the aminoacyl-tRNA synthetase required to charge the tRNA. In order to protect the reactive selenium, as well as create an external mechanism to restore its function in a spatial and temporal fashion, we aim to first protect the reactive selenium with a photolabile protecting group- specifically, an o-nitrobenzyl caging group. By attaching this moiety and inserting the caged UAA into a protein, we hope to exploit the unique properties associated with photocaging amino acids, while at the same time affording potential regulation and enhancement using selenocysteine as a therapeutic agent.

B. Caged UAAs

Proteins have a wide range of biological functions and utilize many mechanisms to regulate these functions. In order to further study these functions, it is beneficial to externally alter or knock-out a protein function to observe the phenotypic effects. Moreover, a non-endogenous mechanism to turn protein function on and off could

possess a high degree of therapeutic value. A highly effective tool for regulation of biological process involves covalently attaching a photolabile group to small organic molecules or amino acids.[11] There are numerous methods and groups used to modify or "cage" small molecules for photoregulation. One popular method involves attachment of a nitrobenzyl group to a biologically active agent, allowing the researcher specific control of the overall function. When the caging group is attached, function will be abbrogated; however, when irradiated with non-cytotoxic UV light, the caging group is removed and function is restored.[12] (Figure 3.2)

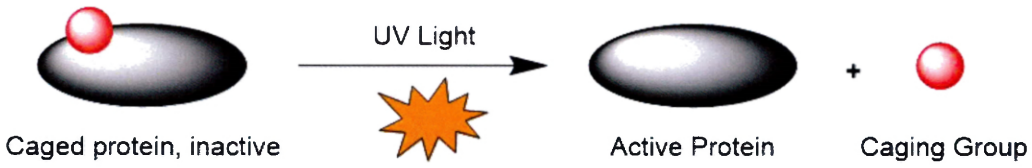


Figure 3.2: When a protein is expressed using a chemically caged amino acid, the protein is rendered inactive. Once exposed to light, however, the caging group detaches, restoring protein activity

Due to the significant biological value of selenocysteine, we propose attaching a nitrobenzyl group to this amino acid (Figure 3.3) in order to both protect the reactive selenium during incorporation as well as further control selenoprotein function once incorporated.

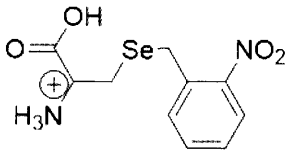
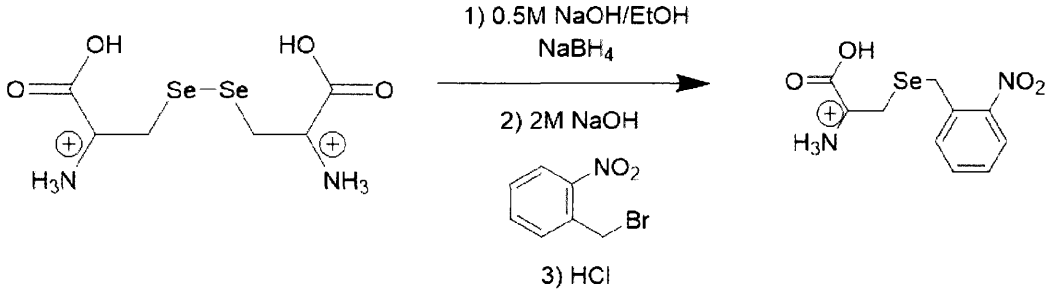


Figure 3.3: Structure of caged Selenocysteine.

II. Synthesis of Caged Selenocysteine

Caged selenocysteine was synthesized based on a previous literature precedent.[4] The synthesis was initiated from the reaction of L-Selenocysteine with a reducing agent, followed by nitrobenzyl-bromide (Scheme 3.1).

Scheme 3.1.



Upon completion of the reaction, solvent was removed from the product *in vacuo* and the product was purified on a silica column using 10:1 DCM:MeOH, then analyzed via ^{1}H NMR. The caged selenocysteine was obtained as a yellow solid in good purity (81%). Moving forward, the focus of this research will shift to incorporating this caged UAA into a model protein via the use of a promiscuous aaRS . Attempts will be made using a wide

array of known synthetases in hopes of finding an already established aaRS that would recognize the selenocysteine UAA to charge the corresponding orthogonal tRNA.

III. Materials and Methods

General. Solvents and reagents were obtained from either Sigma-Aldrich or Fisher Scientific and used without further purification, unless noted. Reactions were conducted under ambient atmosphere with non-distilled solvents. NMR data was acquired on a Varian Gemini 400 MHz.

Synthesis of Caged-Selenocysteine

L-Selenocysteine (20 mg, 0.06 mmol) was dissolved in 320 μL 0.5M NaOH and 80 μL EtOH. Starting material was cooled to 0 °C and sodium borohydride (16 mg, 0.6 mmol) was added to the reaction while stirring. Over the course of addition, the reaction turned from yellow to clear. Reaction was then allowed to reach room temperature, then cooled again to 0°C. Upon cooling, 2M NaOH (160 μL) was added to reaction along with nitrobenzyl-bromide (52 mg, 0.24 mmol). The reaction was allowed to stir at room temperature for an additional 3 hours. Upon completion, the reaction was quenched with HCl to a pH of 6.5. Solvent was then removed *in vacuo* and the product was purified via silica gel column chromatography (DCM:MeOH 10:1) to afford a yellow solid (0.015 g, 0.048 mmol, 81%). ^{1}H NMR (400 MHz; D_2O): δ 7.42-8.05 (m, 8H), 4.15 (s, 2H), 3.85 (t, J=7.0 Hz, 2H), 3.0 (m, 2H).

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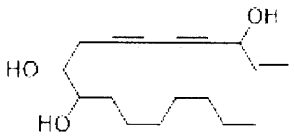
Chapter 4-Introduction to Glaser Hay-Coupling and Solid-Support Synthesis

I. The Polyyne Scaffold

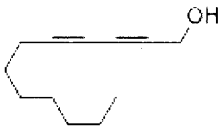
Polyynes are molecules containing two or more conjugated acetylene units. Because of this high degree of π-system conjugation, they are often unstable compounds, but are rich in biological and optical properties.[13] For years, these compounds have been isolated from a wide variety of natural sources such as fungi, coral, plants, and bacteria cultures.[2] They are known to display numerous biological activities including: antibacterial, antifungal, anticancer, and anti-HIV properties.[2] This conjugated acetylenic scaffold can also be observed as a core structure in polymers and other supramolecular materials. The structure gives polymers a versatile linear geometry, which in turn results in molecules with unique optical properties. Siemsen et al. reported that polymers containing polyyne structures and transition metals lead to a unique array of photovoltaic, nonlinear optics, and luminescence properties as well as distinctive crystal structures.[1]

Classically, polyynes are isolated from a wide variety of natural sources. Over one thousand compounds containing these cores have been isolated and shown to exhibit biological activity.[3] (Figure 4.1)



Potential antitumor activity; Originally isolated from ginseng.

Used in Chinese traditional medicine; Originally isolated from *Atractylodes lancea.*



Antibacterial properties; Originally isolated from stony corals.

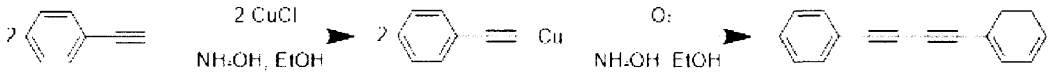
Figure 4.1: Examples of naturally occurring polyyne structures and their uses as natural products.[2]

Many biologically active polyyne molecules have the potential to be prepared in the lab setting. The following research discusses the discovery and optimization of a methodology to efficiently synthesize polyyne molecules in good yield and with minimal steps and purification. It was hypothesized that by utilizing a solid-support, numerous biologically active polyyne molecules could be generated easily and quickly. The described methodology builds on the established Glaser reaction from 1869, and focuses on applying a solid-support system to overcome issues associated with previously described Glaser-Hay reactions. The Glaser-Hay reaction was chosen for testing on the solid-support system due to having a high tolerance of various alkyne functional groups. Moreover, the vast amount of commercially available acetylenic starting material makes using this reaction optimal for combinatorial chemistry, with the potential for creating a diverse library of polyyne compounds.

II. The Standard Glaser Coupling and Further Advancements

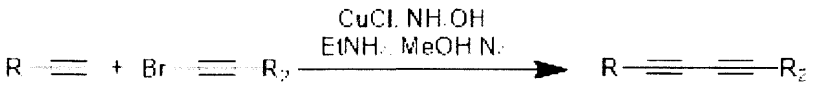
In 1869, synthesis of polyyne core structures was developed by Glaser utilizing the oxidative dimerization of copper (I) phenylacetylide upon exposure to air to yield the conjugated polyyne (Scheme 4.1).[4]

Scheme 4.1.



To further enhance the scope of the Glaser reaction, others have established variant reactions.[5] For example, a new milestone utilizing oxidative acetylenic coupling was discovered by Eglinton and Galbraith in 1956. This protocol coupled acetylenic units by using a copper salt oxidation in methanolic pyridine to provide a homocoupled polyyne.[12] Further, a reaction introduced in 1957, the Cadiot-Chodkiewicz reaction, began to pave the way for synthesis of asymmetrical polyynes. The Cadiot-Chodkiewicz reaction is a cross-coupling reaction utilizing a terminal alkyne/haloalkyne partner and a copper salt/amine base catalyst to provide the desired heterodimer (Scheme 4.2).[6]

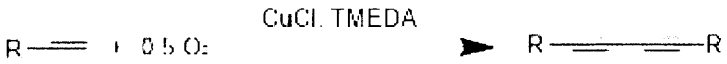
Scheme 4.2



The variant Glaser reaction that provides the basis for this research was established by Hay in 1962. Hay employed catalytic amounts of copper (I) chloride in the

presence of tetramethylenediamine (TMEDA) and oxygen which led to a drastic decrease in reaction time. The resulting Glaser-Hay reaction produced symmetric polyyne molecules in an efficient fashion and afforded higher yields than the standard Glaser reaction (Scheme 4.3).[7]

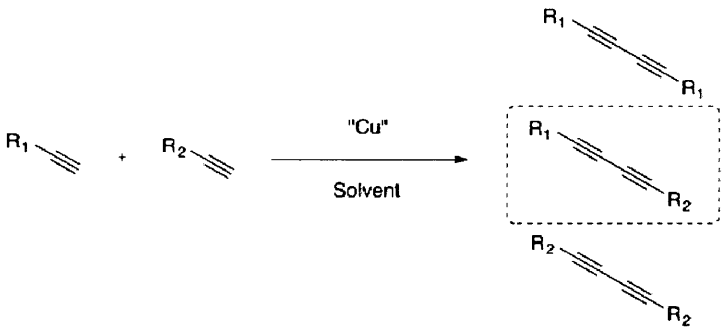
Scheme 4.3



III. Current Glaser-Hay Coupling Reaction Limitations

The Glaser-Hay reaction is a rapid and efficient method to produce symmetrical polyynes from a common terminal alkyne. However, this reaction does not display chemoselectivity when performed with two different terminal alkyne reactants. Using the standard Glaser-Hay conditions while reacting two terminal alkynes yields three different products: two homodimers and a heterodimer (Scheme 4.4). The formation of three products results in tedious further purification and a decreased yield (statistically a 33% yield at best) of the desired heterocoupled product. Additionally, since many of the natural products and biologically active compounds are heterodimers, this reaction is not synthetically useful in their preparation.

Scheme 4.4



Various researchers have employed a solution phase Glaser-Hay reaction using copper acetate or nickel chloride as the catalyst system to produce asymmetrical polyynes. These methodologies are limited to terminal alkyne precursors that will create a homocoupled product that is able to be isolated from the heterocoupled product, which requires extra purification steps and therefore the desired asymmetrical product is only obtained in minimal yield.[8]

IV. Solid Supported Synthesis: Overcoming the standard limitations

Solid-supported synthesis has found a widespread use in organic chemistry, providing an efficient means to produce an expansive number of compounds. Solid supports contain an insoluble polymeric backbone, allowing the attached product to be isolated from the solution phase and affording products in high purity and yield.[14] To date, there is a wide array of solid support systems available, including those with amino,

carboxy, halogen, and hydroxyl functionalities.[15] The type of resin for solid-supported synthesis is chosen based on the desired reaction and the reagents used. Overall, benefits of using solid-supported synthesis include: pseudo-high dilution conditions, the ability to easily wash away excess reagents, and structural rigidity.[14] Therefore, in order to obtain a desired asymmetrical polyyne and address the chemoselectivity issues associated with the Glaser-Hay reaction, we propose immobilizing one of the terminal alkynes on a polystyrene solid support.[9] Once immobilized, the terminal alkyne can be reacted with a different alkyne using the copper catalyst/TMEDA system associated with the standard Glaser-Hay reaction. The solid-support immobilization allows for control over reaction products by allowing the researcher to quickly wash away excess homocoupled product or terminal alkyne reactant while leaving the desired asymmetrical molecule attached to the solid-support. Immobilization of the alkyne also prevents its homodimerization, resulting in pure heterocoupled product upon cleavage from the resin.

Other reactions utilizing a solid-support system have previously been shown to achieve chemoselectivity. One such reaction is the Cadiot-Chodkiewicz reaction which utilizes a terminal alkyne and a haloakyne to produce an asymmetrical polyyne.[6] By immobilizing a haloalkyne on a solid-support and reacting it with a terminal alkyne, researchers were able to eliminate any homodimer formed by the standard reaction.[11]

Although the solid-supported Cadiot-Chodkiewicz reaction was successful, that reaction variant already exhibits a degree of chemoselectivity. Thus, the investigation of a solid-support system for the Glaser-Hay reaction seemed even more advantageous in

that it was a reaction that would not require the synthesis of a haloalkyne precursor, and also demonstrates shorter reaction times and requires fewer post-reaction workup steps, allowing for the efficient synthesis of numerous polyynes.

Even more relevant, Liao et al. described first attempts at Glaser-Hay reactions using a solid-support. Their research, however, was focused on creating a high yield of a specific homocoupled product.[10] Liao et al. utilized the benefits provided by solid-support as well as a AgOTs-$CuCl_2$-TMEDA catalyst system in order to dimerize aliphatic acetylenes (Figure 4.2).[10] The silver (or silver tosylate) in their catalyst system facilitated the coupling, and their work was of vast importance due to the fact that the Glaser-Hay reaction had not been previously performed on solid-support. Ultimately, this group was able to utilize solid-support and their unique AgOTs-$CuCl_2$-TMEDA catalyst system to efficiently produce a small library of homocoupled bis-benzo[b]furan-linked 1,3-diynes.

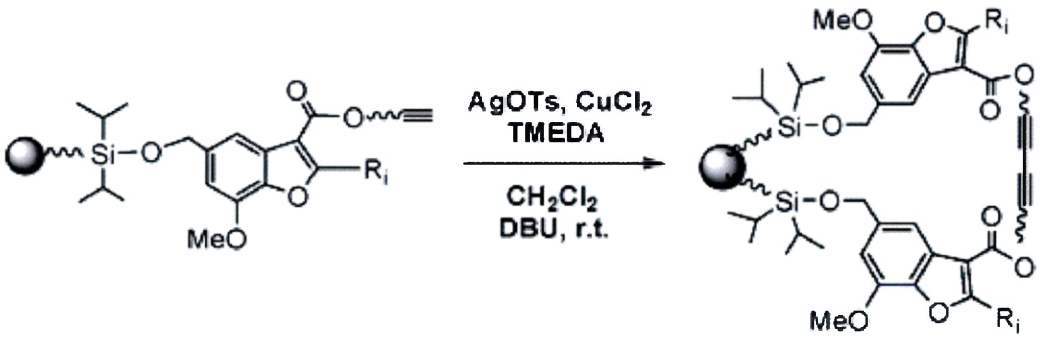


Figure 4.2: Liao et al.'s reaction scheme including their unique catalyst system. This group proposed that Ag along with the typical copper catalyst system would further facilitate the coupling reaction, and result in the quick formation of a pure homocoupled product.[10]

For this research, we hypothesized that a new methodology employing a solid-support to the Glaser-Hay reaction could be created and optimized to afford asymmetric polyynes in high purity and yield. The developed methodology would alleviate the chemoselectivity issues often associated with the standard Glaser-Hay reaction and eliminate the need for tedious post-reaction purification. The solid-supported Glaser-Hay methodology aims to provide an easy synthetic route to the creation of a diverse polyyne library, benefitting the fields of combinatorial, organic, and medicinal chemistry.

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Chapter 5- Solid-Supported Glaser-Hay Couplings

I. Introduction

To alleviate the associated chemoselectivity issues of the Glaser-Hay reaction, we present the development and optimization of solid-supported Glaser-Hay couplings. This novel methodology allows for the rapid preparation of asymmetric polyynes in high purity and yield. Furthermore, fluorescence spectroscopy is used to expose the unique optical properties of these molecules and offer further characterization. Overall, this methodology will benefit the fields of combinatorial, organic, and medicinal chemistry, and provide a quick route to asymmetrical polyynes.

II. Results and Discussion

A. Establishment of Reaction Conditions

Due to the chemoselectivity problems associated with the Glaser-Hay reaction, this reaction was promising for optimization on a solid-supported resin. Also, as it is a widely utilized synthetic reaction, there were a wide array of established reaction conditions in the literature.[1] Reaction conditions were investigated using different solvent and catalyst systems in order to optimize this reaction on a solid support. As a proof-of-concept, phenylacetylene was used for a standard homocoupling Glaser-Hay reaction in solution. Based on literature searches, possible catalyst systems included CuCl/TMEDA, CuI/TMEDA, and CuI/DIEPA/N-bromosuccinimide.[1] Standard reaction conditions (12 hours at room temperature) were carried out with phenylacetylene in solution in order

to test each of these systems. The CuCl/TMEDA system surprisingly did not produce any product, indicating a possible solubility or reactivity problem within this reaction. Furthermore, the CuI/DIEPA/N-bromosuccimide system only produced trace amounts of product, possibly due to negative interactions with the polystyrene core of the resin. Gratifyingly, the CuI/TMEDA was found to produce a high yield of homocoupled phenylacetylene, indicating that this would be an ideal catalyst system to use for the solid-supported Glaser-Hay reactions.

B. Resin Optimization

Initially, three different types of polystyrene resins were investigated: carboxy, trityl chloride, and bromo derivatized supports. Overall, the trityl chloride resin was chosen based on having high reproducibility with alkyne loading and mild alkyne immobilization and cleaving conditions. Propargyl alcohol (10 equiv.) was first chosen for immobilization on the resin, and was reacted at room temperature for 16 hours with the trityl chloride resin and triethylamine (10 equiv.). This afforded a propargyl alcohol loaded resin that could then be employed in Glaser-Hay reactions. Phenylacetlyene was the terminal alkyne used to optimize coupling conditions based on the ability to easily observe the heterodimeric products via ^{1}H NMR and TLC. Solvents, temperatures, and the loading of the resin were all varied to determine optimal conditions.

Several solvents were examined, including toluene, THF, DCM, and acetonitrile. Alkynes were reacted in each solvent for 12 hours at room temperature, and the yield of

product was determined following resin cleavage. THF was chosen due to the fact that it reproducibly afforded the highest yields, and all other solvents resulted in substantial amounts of unreacted starting material. To further optimize reaction conditions, reaction temperature was then varied, with 30 °C, 60 °C, and 80 °C all tested. Reactions conducted at 30 °C did not provide sufficient heat for the reaction to progress, and afforded no yield of product. The high temperature of 80 °C was found to drive the reaction to completion; however, product yields were low, potentially due to the high temperature leading to resin degradation. When performed at 60 °C, yields were found to be favorable, and therefore this temperature was selected as optimal for this reaction.

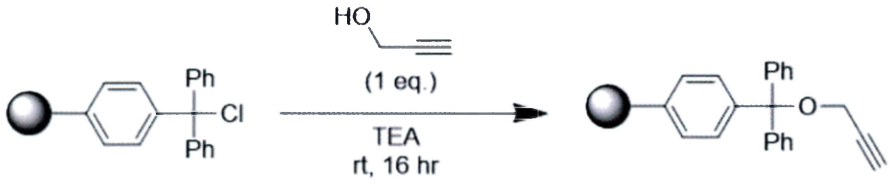
Additionally, it was demonstrated that resin loading conditions played a critical role in optimization of the solid supported reaction. When a propargyl alcohol derivatized resin was reacted with phenylacetylene for 12 hours at 60 °C, homodimerization of the immobilized propargyl alcohol was discovered. This formation resulted in a lower reaction yield, and failed to eliminate homocoupled product, which was the overall goal of the immobilization. Also, further purification was then needed to eliminate the undesired product. Therefore, further optimization was necessary in order to obtain asymmetrical polyynes in good yield and without the formation of the undesired homodimer.

To alleviate this undesired coupling product, the initial loading of propargyl alcohol onto the resin was investigated. Due to the formation of propargyl alcohol homodimer, it was believed that decreasing the loading of propargyl alcohol onto the resin would reduce immobilization proximity and prevent homodimerization. Initially,

propargyl alcohol was loaded onto the polystyrene resin in 10 equivalents with TEA (10 equiv.), affording a resin loading value of 1 mmol/g. This had resulted in maximum loading of the starting material and ultimately led to homodimerization issues. To decrease loading values, amount of starting material and immobilization reaction time were lowered and loading was assessed.

Resins were then immobilized using a range of conditions. By using a trityl chloride resin with 10 equiv. of propargyl alcohol and 10 equiv. of TEA and reacting at 40 °C for 16 hour a higher loaded resin of ~1.8 mmol/g was achieved. Another reaction using same amounts of reagents at the same temperature, but only a 2 hour reaction time was also set up and afforded a ~1.0 mmol/g medium loaded resin. Finally, the amount of starting propargyl alcohol was decreased to produce a low loaded resin of ~0.6 mmol/g (Scheme 5.1). Loading conditions could be quantitated via cleavage of the immobilized resin with 2% trifluoroacetic acid, and analysis of resin loadings based on mass recovery and GC/MS values found for propargyl alcohol.[2]

Scheme 5.1



The derivatized resins were then assessed using the previously optimized reaction conditions for the solid-supported Glaser-Hay coupling (60 °C for 16 hr.). The loading

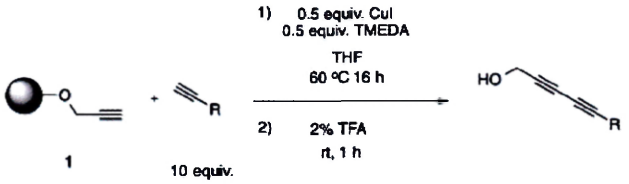
proved to have significant effect on the product formation, as the resins loaded above 0.7 mmol/g were found to produce the undesired homodimer. Satisfyingly, the lower loaded resin eliminated the homodimer formation, and could still be used to efficiently produce the desired asymmetrical polyynes.

C. Preparation of a Diverse Diyne Library

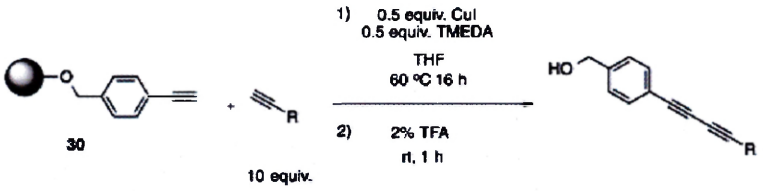
With solid-supported Glaser-Hay reaction conditions optimized, the methodology could be applied to a plethora of terminal alkynes in order to afford a diverse diyne library. Propargyl alcohol and 4-ethynyl benzyl alcohol were immobilized on the trityl choride resin to afford resin loadings of 0.6 mmol/g.

Optimization experiments demonstrated that ideal conditions to maximize both yield and purity were as follows: reacting for 16 hours at 60 °C in tetrahydrofuran with a CuI/TMEDA catalyst system. (Scheme 5.2 and 5.3) Upon completion, the reactions were filtered and washed with alternating rinses of DCM and MeOH. These washes were employed in order to remove the excess unreacted starting material and any homodimer that resulted from the undesired reaction of the soluble alkyne with itself. The filtered and washed resin was then dried and cleaved with 2% trifluoroacetic acid. All solvent was easily removed *in vacuo*, and the desired product was analyzed via ^{1}H NMR and GC/MS.

Scheme 5.2



Scheme 5.3



We next employed these conditions with a variety of terminal alkynes. Aromatic rings, silyl groups, amines, alcohols, and alkyl chains were all assessed in the solid-supported Glaser-Hay reactions to provide a diverse set of diyne products. (Tables 5.1 and 5.2)

Table 5.1: Diynes synthesized using immobilized propargyl alcohol resin.

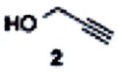
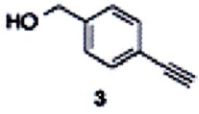
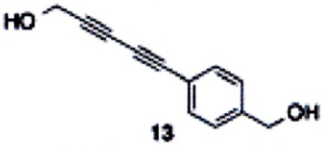
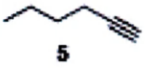
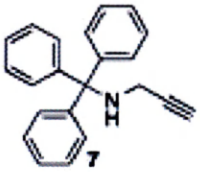
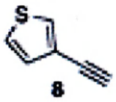
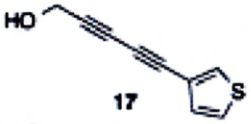
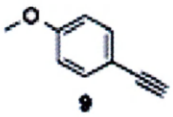
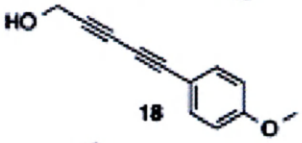
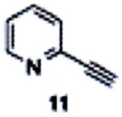
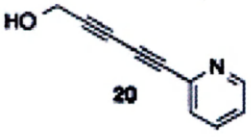
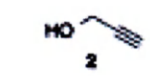
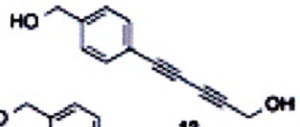
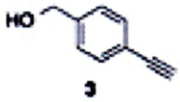
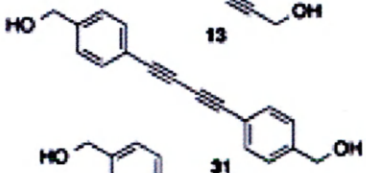
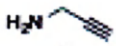
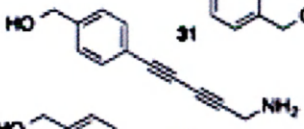
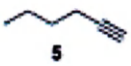
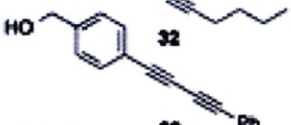
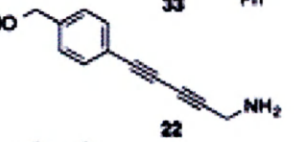
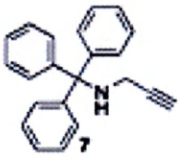
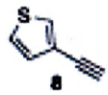
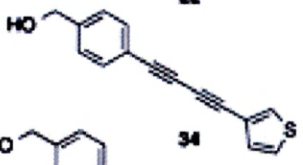
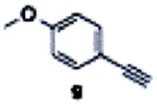
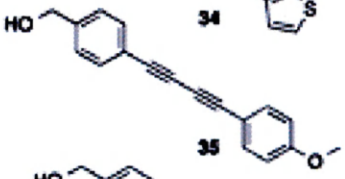
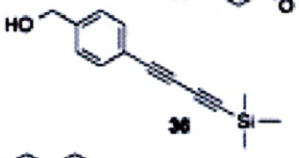
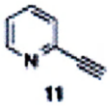
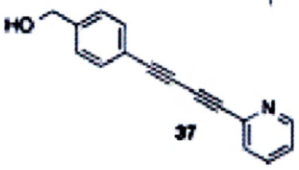
Alkyne	Product	% Yield
 2	 12	99%
 3	 13	56%
 4	 14	40%
 5	 15	84%
 6	 16	95%
 7	 14	91%
 8	 17	45%
 9	 18	99%
 10	 19	80%
 11	 20	55%

Table 5.2: Diynes synthesized using 4-ethynyl benzyl alcohol immobilized resin.

Alkyne	Product	% Yield
 2	 13	45%
 3	 31	99%
 4	 22	98%
 5	 32	98%
 6	 33	98%
 7	22	99%
 8	 34	42%
 9	 35	73%
 10	 36	87%
 11	 37	53%

During the analysis of different terminal alkynes, it was discovered that the alkynes containing nucleophilic species, such as amine, benzyl alcohol, thiophene, and pyridine, often resulted in lower reaction yields. These nucleophilic species could potentially interact with the copper catalyst system, therefore interfering in its ability to yield diyne and lowering the overall yield. It was elucidated that the lower yields due to nucleophilic reactants could be avoided by protecting the nucleophilic site with a trityl group. (Affording Product **7**) Moreover, the protecting group can be easily removed with the 2% TFA cleavage step obviating the necessity for an additional deprotection.

To further enhance the scope of this methodology, the resin was also loaded with propargyl amine. (Scheme 5.4). The propargyl amine resin was shown to undergo the same reactions with terminal alkynes under identical reaction conditions. Overall, this allowed for the preparation of the same scaffold generated from the alcohol loaded resins, but now with the amine functionality present. (Table 5.3)

Scheme 5.4

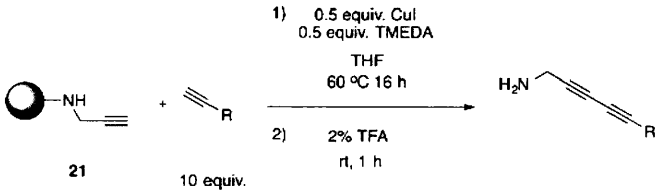
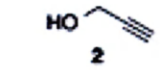
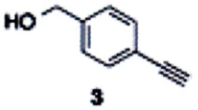
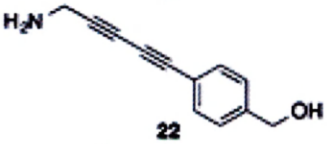
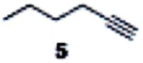
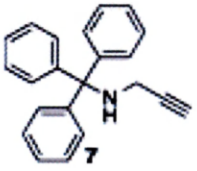
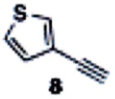
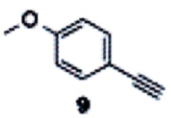
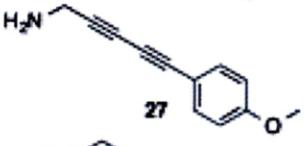
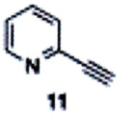


Table 5.3: Diynes synthesized using propargyl amine immobilized resin.

Alkyne	Product	% Yield
 2	 14	86%
 3	 22	78%
 4	 23	87%
 5	 24	71%
 6	 25	92%
 7	 23	98%
 8	 26	48%
 9	 27	79%
 10	 28	84%
 11	 29	41%

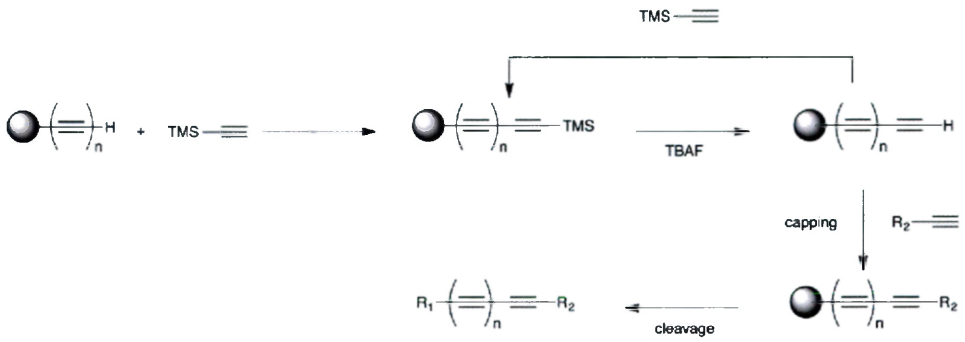
Overall, this work demonstrates that the solid-supported Glaser-Hay methodology can be employed with a diverse set of terminal alkynes in order to afford a variety of asymmetrical diynes, increasing yields and solving the standard chemoselectivity issues associated with the Glaser-Hay reaction. These reactions required little reaction work-up or purification and resulted in good yields.

D. Extending the Technology Towards the Synthesis of Polyynes

With the solid-supported Glaser-Hay reaction optimized and diyne library prepared, this research transitioned to extend the carbon backbone of the acetylenic scaffold and create a similar library of polyyne molecules including triynes, tetraynes, and pentaynes. By doing so, this methodology could be used to synthesize a wider array of biologically relevant molecules. Furthermore, upon extended conjugation these molecules have potential as highly conductive molecular wires.

To begin this extended methodology, we are able to utilize molecules made in the initial asymmetrical diyne libraries. The diyne library includes three asymmetrical diyne molecules that involved reacting the immobilized terminal alkyne with trimethylsilylacetylene (TMS acetylene). Fortuitously, these molecules can undergo a silyl deprotection by reacting with the nucleophilic fluorine species in tetra-*n*-butylammonium fluoride (TBAF). The TMS cleavage regenerates a terminal alkyne that can be further reacted in the Glaser-Hay coupling with an additional terminal alkyne or can further be extended by another addition and cleavage of TMS. (Scheme 5.5)

Scheme 5.5



Desilylation in order to prepare polyynes had been previously reported by Heuft et. al. Here, a copper catalyst system was also utilized, and addition and cleavage of TMS enabled the researchers to create symmetrical polyynes.[3] By using this methodology and applying it to the optimized solid-supported Glaser-Hay reaction, the previously prepared asymmetrical diyne library could be further extended and diversified.

E. Preparation of a Diverse Polyyne Library

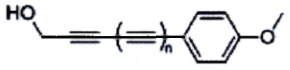
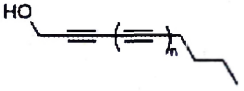
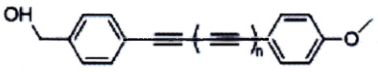
To prepare a polyyne library, the trityl-chloride polystyrene resin was loaded as previously described with either propargyl alcohol or 4-ethynyl benzyl alcohol. These were chosen due to previous success producing diynes in high yield using a solid support. Loaded resin was reacted under optimized solid-supported Glaser-Hay conditions with TMS acetylene. The resin was then washed and dried as described previously. Next, the resin was subjected to silyl cleavage using a 1 M TBAF solution for 1 hour at room temperature. Upon cleavage the resin was again washed 5x with DCM and MeOH, and dried. This afforded a regenerated terminal alkyne on the resin that could either be

capped with another terminal alkyne or undergo another round of TMS acetylene addition/cleavage.

Initially, 4-ethynylanisole was chosen for the capping alkyne due to its unique NMR signature and easy visualization by TLC via its UV activity. Upon capping, resin was cleaved with the 2% TFA solution, resulting in the preparation of a polyyne. The first capping produced the triyne of propargyl alcohol and 4-ethynylanisole in good yield (68%) and required no further purification. The product was then analyzed via TLC, ^{1}H NMR, and GC/MS. This initial product demonstrated that the previously optimized methodology could be used to produce a small library of polyyne molecules.

Loaded resin underwent numerous rounds of TMS acetylene coupling/TBAF deprotection and terminal alkyne capping to afford a diverse array of tryiynes, tetraynes, and pentaynes. 4-Ethynyl benzyl alcohol was also utilized to afford two diverse sets of polyyne scaffolds. A variety of terminal alkynes were used for capping and the diverse array of synthesized polyynes can be seen in Table 5.4.

Table 5.4: Polyyne Library

Compound	n	Product #	Yield
	1	18	99%
	2	38	68%
	3	39	67%
	4	40	44%
	1	14	45%
	2	41	46%
	3	42	46%
	4	43	48%
	1	16	95%
	2	44	63%
	3	45	63%
	4	46	56%
	1	15	84%
	2	47	53%
	3	48	38%
	4	49	41%
	1	12	99%
	2	50	82%
	3	51	57%
	4	52	49%
	1	20	55%
	2	53	34%
	3	54	30%
	4	55	27%
	1	35	73%
	2	56	56%
	3	57	52%
	4	58	46%

Products of the solid-supported reaction needed little (via a quick silica plug) to no purification, and were obtained in moderate to good yield. Unfortunately, yet not surprisingly, as the number of synthetic steps increased, the yields decreased. However, the yields were still rather high given the number of synthetic manipulations. Products could be easily characterized via TLC, ^{1}H NMR, and GC/MS.

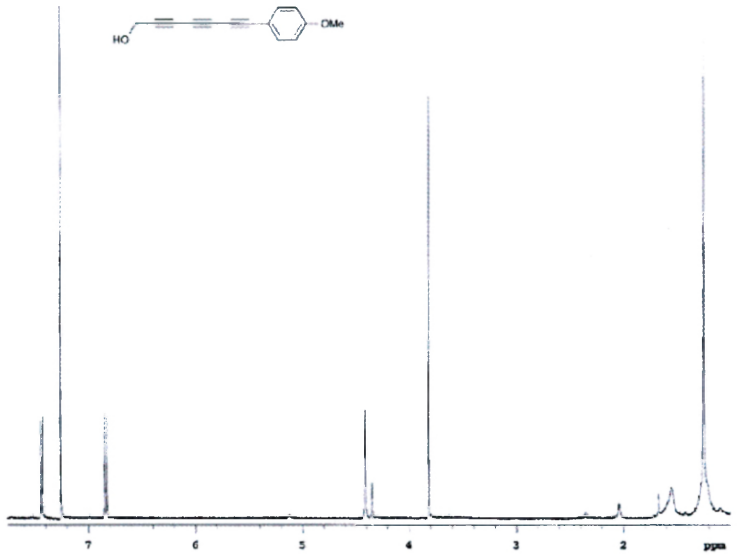


Figure 5.1: Selected sample ^{1}H NMR taken in $CDCl_3$ showing compound purity (Compound 38).

This extension of the previously reported methodology shows the versatility and usefulness of the solid-supported Glaser-Hay reaction. A library of triynes, tetraynes, and pentaynes was synthesized efficiently and could be further studied for unique optical properties. Additionally, these molecules can be assessed in the future for any biological activity they may exhibit.

F. Fluorescence Properties of Polyyne Library

Polyynes are known to exhibit unique optical properties due to their substantial amount of conjugation. This often results in molecules that may act as molecular wires due to their geometric and conducing properties.[4,5] Therefore, to elucidate these unique properties, the absorbance and fluorescence spectra for these series of molecules were next observed and analyzed. Spectra were obtained using a PerkinElmer LS 55 Luminescence Spectrometer at an excitation wavelength of 330 nm. The fluorescent shifts of the different capped alkynes within a series of triynes, tetraynes, or pentaynes was studied. The terminal alkynes that contained additional conjugation, such as phenylacetylene, exhibited a stronger fluorescence intensity than those containing only alkyl sidechains such as hexyne. The difference in intensity and wavelength can be viewed throughout each series. (Figures 5.2, 5.3, and 5.4)

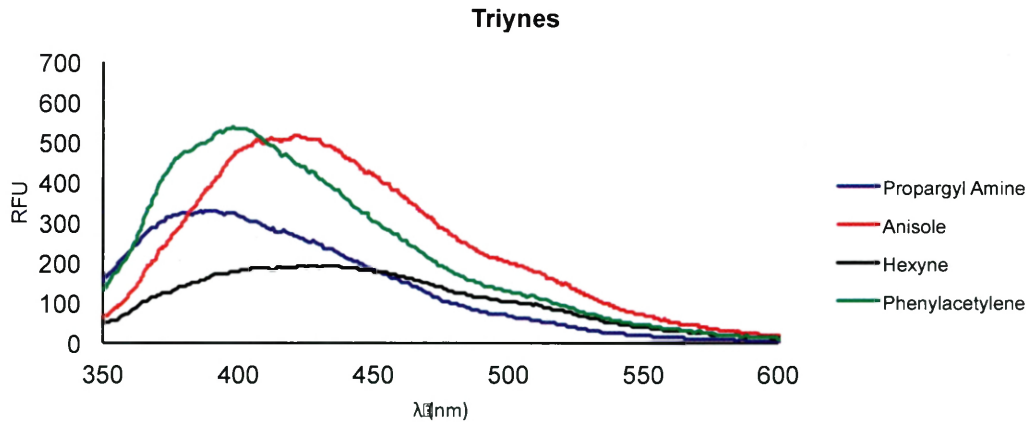


Figure 5.2: Fluorescence spectra for triynes, excited at 330 nm. Product solutions were 5 mM dissolved in DCM.

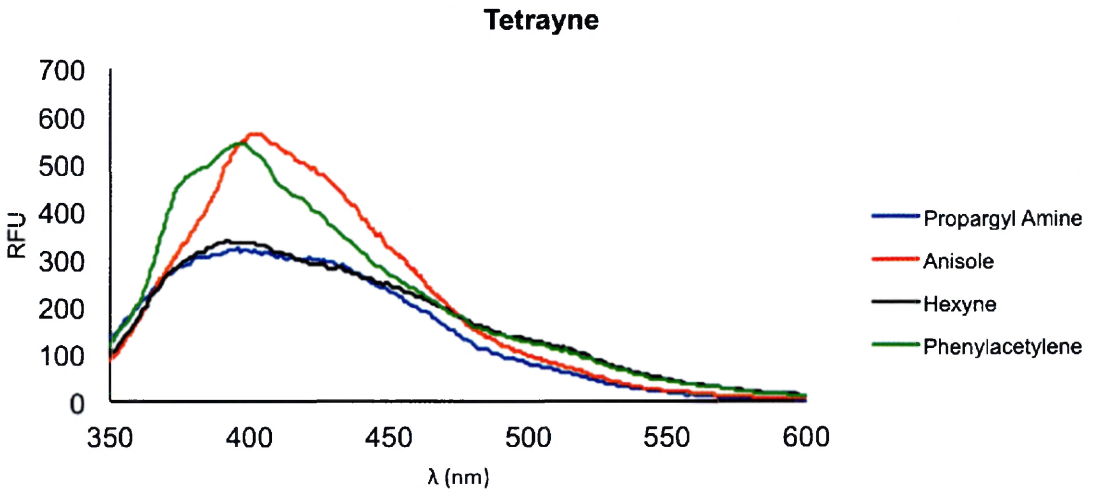


Figure 5.3: Fluorescence spectra for tetraynes, excited at 330 nm. Product solutions were 5 mM dissolved in DCM.

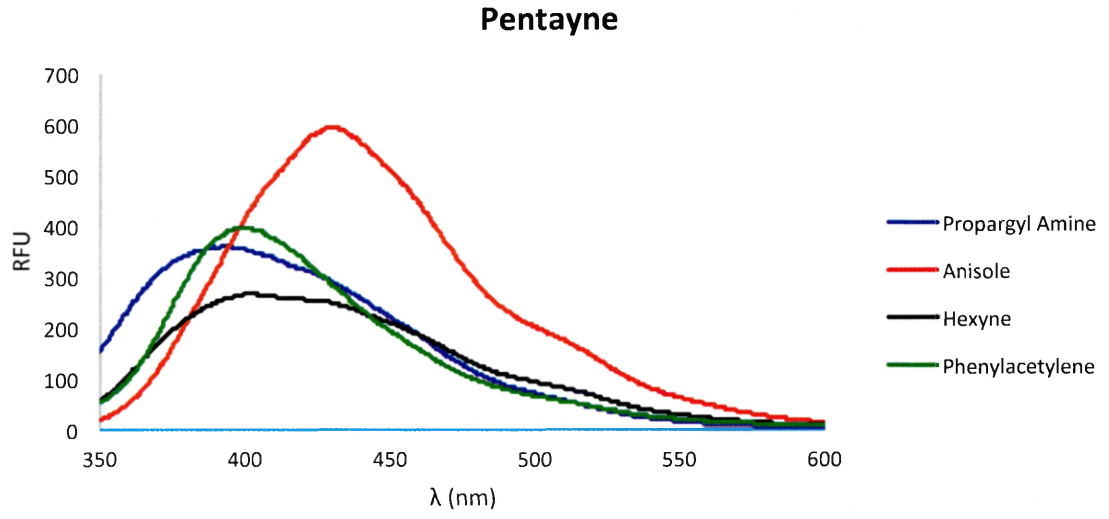


Figure 5.4: Fluorescence spectra for penatynes, excited at 330 nm. Product solutions were 5 mM dissolved in DCM.

To further investigate and understand how the shifts correlated to conjugation and the extension of the acetylenic scaffold, 4-ethynyl benzyl alcohol was also loaded and reacted under standard conditions with ethynylbenzyl alcohol. The fluorescence spectra of this series can be seen in (Figure 5.5). This series shows a dramatic blue shift exhibited by the pentayne.

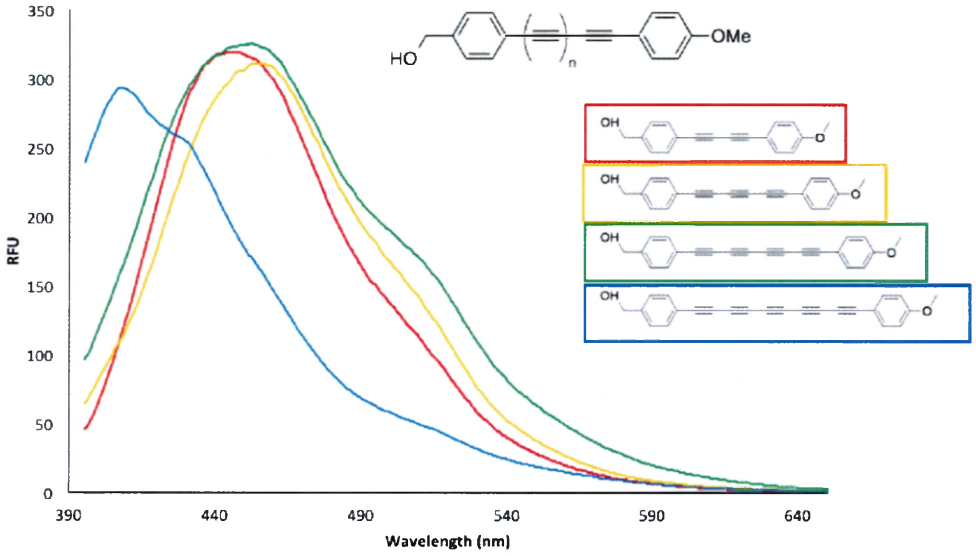


Figure 5.5: Fluorescence spectra when excited at 370 nm for 4-ethynyl benzyl alcohol reacted with ethynylbenzylanisole. Product solutions were 5 mM dissolved in DCM.

Upon analysis of the fluorescence spectra it was found that there are some common themes; however, the overall shifts among the polyynes are difficult to predict. As the conjugation due the extended acetylenic scaffold increases, the diyne, triyne, and tetrayne displayed a slight red shift from the previous molecule. When the pentayne was

examined, the spectra did not follow this pattern, and displayed a drastic blue shift from the others. It was hypothesized that upon the extension of the highly conjugated core these molecules may transition into a polymer-like state. Therefore, the spectra of the highly conjugated pentaynes could be dramatically and unpredictably shifted from the spectra of the triynes. Literature precedent corroborated that spectral shifts of these molecules are hard to predict, but that their unique spectra can still be of use to further study and characterize these molecules.[6] Through the use of fluorescence spectroscopy, we are able to expose these molecules unique optical properties and offer insight into further molecule characterization.

III. Materials and Methods

General. Solvents and reagents were obtained from either Sigma-Aldrich or Fisher Scientific and used without further purification, unless noted. Tritylchloride resin, 100-200 mesh, 1% DVB crosslinking, was purchased from Advanced Chemtech. Reactions were conducted under ambient atmosphere with non-distilled solvents. NMR data was acquired on a Varian Gemini 400 MHz. GC/MS analysis was conducted on an Agilent Technologies 6890N GC system interfaced with a 5973N mass selective detector. An Agilent J&W GC capillary column (30 m length, 0.32 mm diameter, 0.25 nm film) was employed with a splitless injection (250 °C inlet, 8.8 psi) with an initial 70 °C hold (2 min) and ramped for 15 min to 230 °C. Fluorescence data was measured using a PerkinElmer LS 55 Luminescence Spectrometer.

Immobilization of Alcohol onto Trityl Chloride Resin in Low Loading Conditions

Trityl chloride resin (200 mg, 0.36 mmol, 1 equiv.) and dichloromethane (5 mL) were

added to a flame dried vial. The resin was swelled at room temperature with gentle stirring for 15 min. Alcohol (25.0 μL, ~1.2 equiv.) was added to the reaction, followed by triethylamine (10.0 μL, 0.072 mmol, 0.2 equiv). The mixture was stirred at room temperature for 16 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each). The resin was swelled in CH_2Cl_2 and dried under vacuum for 45 min before further use. Resin loading was assessed via cleavage of 15 mg resin in 2% TFA in DCM (200 πL) at room temperature for 1 hour. After filtration of resin, loading was determined both by mass of cleaved product and by GC/MS analysis of integrated alcohol peaks from a standard curve and the cleaved resin.

Sample of Glaser Hay Coupling Protocol at 60 °C

Soluble alkyne (0.350 mmol, 10 equiv.) was added to a flame dried vial containing the propargyl alcohol derivatized trityl resin (50 mg, 0.035 mmol, 1 equiv.), and tetrahydrofuran (0.750 mL). The copper catalyst (10 mg, 0.053 mmol) and tetramethylethylenediamine (10 μL, 0.066 mmol) were added to a separate flame-dried vial then dissolved in tetrahydrofuran (0.750 mL). The catalyst mixture was then added to the resin in one portion and stirred at 60 °C for 16 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each). The product was then cleaved from the resin by treatment with 2% TFA (DCM, 1 h) and filtered into a vial. If necessary, a short silica plug was utilized to remove unreacted starting material (1:1 EtOAc/Hex).

Polyyne Extension Protocol

Trimethylsilylacetylene (160 μL, 1.05 mmol, 15 equiv.) was added to a flame dried vial containing the alcohol derivatized trityl resin (100 mg, 0.07 mmol, 1 equiv.), and tetrahydrofuran (2.0 mL). The copper catalyst (20 mg, 1.06 mmol) and tetramethylethylenediamine (20 μL, 0.132 mmol.) were added to a separate flame-dried vial then dissolved in tetrahydrofuran (2.0 mL). The catalyst mixture was then added to

the resin in one portion and stirred at 60 °C for 16 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each).

Regeneration of Terminal Alkyne

The TMS group was then cleaved by incubation in 1 M tetra-n-butylammonium fluoride trihydrate in DCM (TBAF, 1 mL, 1 h). Then the reaction was again transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each) and dried under vacuum for 45 minutes. Product was weighed and transferred to flame dried vial for future use.

Polyyne Capping

Soluble alkyne (15 equiv.) was added to a flame dried vial containing the desired alcohol derivatized trityl resin and tetrahydrofuran (2 mL). The copper catalyst (20 mg, 1.06 mmol) and tetramethylethylenediamine (20 μL, 0.132 mmol) were added to a separate flame-dried vial then dissolved in tetrahydrofuran (2 mL). The catalyst mixture was then added to the resin in one portion and stirred at 60 °C for 16 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each). The product was then cleaved from the resin by treatment with 2% TFA (DCM, 1 h) and filtered into a vial. If necessary, a short silica plug was utilized to remove unreacted starting material (1:1 EtOAc/Hex).

Analytical Data: Diyne NMR Listings

Compound 12: The solvent was removed *in vacuo* to give compound 12 as a white solid (5 mg, 0.032 mmol, 99%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 4.36 (s, 4H); GCMS (Rt=7.88 min) calculated for CH_6O_2 110.0, found 110.1.

Compound 13: The solvent was removed *in vacuo* to give compound 13 as a solid (3mg, 0.020 mmol, 56%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.49 (d, J = 8.0 Hz, 2H), 7.44 (d, J = 8.0 Hz, 2H), 4.72 (s, 2H), 4.35 (s, 2H); GCMS (Rt=9.94 min) calculated for $C_{12}H_{10}O_2$ 186.1, found 186.1.

Compound 14: The solvent was removed *in vacuo* to give compound 14 as a solid (3 mg, 0.014 mmol, 40%). Using 7 as a starting material (3.5 mg, 0.032mmol, 91%). ^{1}H NMR (400 MHz; CD_3OD): δ 5.21 (s, 2H), 4.35 (s, 2H), 3.39 (s,2H); GCMS (Rt=6.01 min) calculated for C_6H_7NO 109.1, found 109.1.

Compound 15: The solvent was removed *in vacuo* to give compound 15 as a solid (4 mg, 0.029 mmol, 84%) ^{1}H NMR (400 MHz; $CDCl_3$): δ 4.32 (s, 2H), 2.29 (t, J = 6.8 Hz,3H), 1.52 (m, 2H), 1.42 (sextet, J = 7.2 Hz, 2H), 0.91 (t, J = 7.23 Hz,3H); GCMS (Rt=7.29 min) calculated for $C_9H_{12}O$ 136.1, found 136.0.

Compound 16: The solvent was removed *in vacuo* to give compound 16 as a solid (4 mg, 0.034 mmol, 95%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.49 (t, J = 5.9, 2H), 7.34-7.26 (m, 3H), 4.45 (s, 2H), 1.94 (s, 1H); GCMS (Rt=9.20 min) calculated for $C_{11}H_8O$ 156.1, found 156.2.

Compound 17: The solvent was removed *in vacuo* to give compound 17 as a solid (2 mg, 0.015 mmol, 45%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.58 (d, J = 4.7 Hz, 1H), 7.28 (s,1H), 7.15 (d, J = 4.7 Hz, 1H), 4.42 (s, 2H); GCMS (Rt=9.67 min) calculated for C_9H_6OS 162.0, found 162.1.

Compound 18: The solvent was removed *in vacuo* to give compound 18 as a solid (6 mg, 0.035 mmol, 99%) ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.44 (d, J = 7.6 Hz, 2H), 6.85 (d, J = 7.6, 2H), 4.14 (s, 2H), 3.82 (s, 3H); GCMS (Rt=9.93 min) calculated for $C_{12}H_{10}O_2$ 186.1, found 186.2.

Compound 19: The solvent was removed *in vacuo* to give compound 19 as a solid (4 mg, 0.026 mmol, 80%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 4.33 (s, 2H), 0.20 (s, 9H); GCMS (Rt=9.79 min) calculated for $C_8H_{12}OSi$ 152.1, found 152.1.

Compound 20: The solvent was removed *in vacuo* to give compound 20 as a solid (2 mg, 0.019 mmol, 55%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.01 (d, J = 7.5 Hz, 1H), 7.65 (t, J = 7.4 Hz, 1H), 7.32 (t, J = 7.5 Hz, 1H), 6.91 (d, J = 7.4 Hz, 1H), 4.38 (s, 2H). GCMS (Rt=8.77 min) calculated for $C_{10}H_7NO$ 157.1, found 157.2

Compound 22: The solvent was removed *in vacuo* to give compound 22 as a solid. (4 mg, 0.022 mmol, 76%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.40 (d, J=7.5 Hz, 2H), 7.33 (d, J=7.5 Hz, 2H), 4.61 (s, 2H), 3.28 (s, 2H). GCMS (Rt=9.92 min) calculated for $C_{12}H_{11}NO$ 185.1, found 185.2.

Compound 23: The solvent was removed *in vacuo* to give compound 23 as a solid (3 mg, 0.024 mmol, 87%). ^{1}H NMR (400 MHz; CD_3OD): δ 5.12 (s, 4H), 3.39 (s, 4H); GCMS (Rt=5.72 min) calculated for $C_6H_8N_2$ 108.1, found 108.1.

Compound 24: The solvent was removed *in vacuo* to give compound 24 as a solid (3 mg, 0.019 mmol, 71%). ^{1}H NMR (400 MHz; CD_3OD): δ5.24 (s, 2H), 3.41 (s, 2H), 2.29 (t, J = 6.8 Hz, 3H), 1.52 (m, 2H), 1.42 (m, 2H), 0.91 (t, J = 6.8 Hz, 3H); GCMS (Rt=7.19 min) calculated for $C_9H_{13}N$ 135.1, found 135.2.

Compound 25 The solvent was removed *in vacuo* to give compound 25 as a solid (4 mg, 0.026 mmol, 92%). ^{1}H NMR (400 MHz; CD_3OD): δ 7.49 (t, J = 5.9, 2H), 7.34-7.26 (m,3H), 5.21 (s, 2H), 3.35 (s, 2H); GCMS (Rt=8.14 min) calculated for $C_{11}H_9N$ 155.1, found 155.2.

Compound 26: The solvent was removed *in vacuo* to give compound 26 as a solid (2 mg, 0.013 mmol, 48%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.58 (d, J = 4.7 Hz, 1H), 7.28 (s,1H), 7.15 (d, J = 4.7 Hz, 1H), 5.21 (s, 2H), 3.35 (s, 2H); GCMS (Rt=9.59 min) calculated for C_9H_7NS 161.0, found 161.2.

Compound 27: The solvent was removed *in vacuo* to give compound 27 as a solid (4 mg, 0.022 mmol, 79%). ^{1}H NMR (400 MHz; CD_3OD): δ 7.34 (d, J = 7.6, 2H), 6.75 (d, J= 7.6, 2H), 5.14 (s, 2H), 3.82 (s, 3H), 3.39 (s, 2H); GCMS (Rt=9.82min) calculated for $C_{12}H_{11}NO$ 185.1, found 185.1.

Compound 28: The solvent was removed *in vacuo* to give compound 28 as a solid. (4 mg, 0.023 mmol, 84%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 0.15 (s, 9H), 3.31 (s, 2H); GCMS (Rt=9.87 min) calculated for $C_8H_{13}NSi$ 151.1, found 151.1

Compound 29: The solvent was removed *in vacuo* to give compound 29 as a solid. (2 mg, 0.011 mmol, 41%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.90 (t, J=7.5 Hz, 1H), 7.65 (d, J=7.4 Hz, 1H), 7.6(d, J=7.4 Hz, 1H), 7.5 (t, J=7.4 Hz, 1H), 3.2 (s, 2H); GCMS (Rt=8.04 min) calculated for $C_{10}H_8N_2$ 156.1, found 157.2.

Compound 31: The solvent was removed *in vacuo* to give compound 31 as a solid. (11 mg, 0.044 mmol, 99%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.55 (d, J=7.3 Hz, 4H), 7.35 (d, J=7.3 Hz, 4H), 4.75 (s, 4H). GCMS (Rt=14.78 min) calculated for $C_{18}H_{14}O_2$ 262.1, found 262.0.

Compound 32: The solvent was removed *in vacuo* to give compound 32 as a solid. (9 mg, 0.043 mmol, 98%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.52 (d, J=7.5 Hz, 2H), 7.31 (d, J=7.4 Hz, 2H), 4.73 (s, 2H), 2.32 (t, J=7.1 Hz, 2H), 1.56 (quintet, J=7.1 Hz, 2H), 1.42 (m, 2H), 0.95 (t, J=7.1 Hz, 3H); GCMS (Rt=9.85 min) calculated for $C_{15}H_{16}O$ 212.1, found 212.2.

Compound 33: The solvent was removed *in vacuo* to give compound 33 as a solid. (9 mg, 0.042 mmol, 32%). 1H NMR (400 MHz; $CDCl_3$): δ 7.53 (d, J=7.5 Hz, 4H), 7.45 (t, J=7.5 Hz, 4H), 7.30 (m, J=7.5, 5H), 4.74 (s, 2H); GCMS (Rt=10.75 min) calculated for $C_{17}H_{12}O_2$ 232.1, found 232.1.

Compound 34: The solvent was removed *in vacuo* to give compound 34 as a solid. (4 mg, 0.018 mmol, 42%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.51 (d, J=7.5 Hz, 2H), 7.31 (d,J=7.4 Hz, 2H), 7.26 (s, 1H), 7.19 (d, J=2.8 Hz, 1H), 7.10 (d, J=2.8 Hz, 1H), 4.74 (s, 2H); GCMS (Rt=9.57 min) calculated for $C_{15}H_{10}OS$ 238.1, found 237.9.

Compound 35: The solvent was removed *in vacuo* to give compound 35 as a solid. (8 mg, 0.032 mmol, 73%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.53 (d, J=7.5 Hz, 2H), 7.30 (d,J=7.5 Hz, 2H), 7.40 (d, J=7.5 Hz, 2H), 6.95 (d, J=7.5 Hz, 2H), 4.65 (s, 2H), 3.96 (s, 3H); GCMS (Rt=13.10 min) calculated for $C_{18}H_{14}O_2$ 262.1, found 262.1.

Compound 36: The solvent was removed *in vacuo* to give compound 36 as a solid. (9 mg, 0.038 mmol, 87%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.52 (d, J=7.3 Hz, 2H), 7.32 (d, J=7.3 Hz, 2H), 4.76 (s, 2H), 0.27 (s, 9H); GCMS (Rt=9.41 min) calculated for $C_{14}H_{16}OSi$ 228.1, found 228.2.

Compound 37: The solvent was removed *in vacuo* to give compound 37 as a solid. (5 mg, 0.023 mmol, 53%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.61 (t, J=8.6 Hz, 1H), 7.55 (t,J=8.6 Hz, 1H), 7.53 (d, J=7.3 Hz, 2H), 7.40 (d, J=7.4 Hz, 1H), 7.31 (d, J=7.3 Hz, 2H), 7.25 (s, 1H), 4.65 (s, 2H); GCMS (Rt=10.75 min) calculated for $C6H_{11}NO$ 233.1, found 233.3.

Analytical Data: Polyyne NMR Listings

Compound 38: The solvent was removed *in vacuo* to give 38 as a solid (10 mg, 0.049 mmol, 68%) ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.44 (d, J = 7.6 Hz, 2H), 6.86 (d, J = 7.6 Hz, 2H), 4.38 (s, 2H), 3.82 (s, 3H); GCMS (R_t =11.02 min) calculated for $C_{14}H_{10}O_2$ 210.2, found 210.3.

Compound 39: The solvent was removed *in vacuo* to give 39 as a solid (11 mg, 0.037 mmol, 67%) ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.46 (d, J = 7.6 Hz, 2H), 6.84 (d, J = 7.6 Hz, 2H), 4.38 (s, 2H), 3.82 (s, 3H); GCMS (R_t = 18.39 min) calculated for $C_{16}H_{10}O_2$ 234.3, found 234.2.

Compound 40: The solvent was removed *in vacuo* to give 40 as a solid (8 mg, 0.031 mmol, 44%) ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.42 (d, J = 7.6 Hz, 2H), 6.85 (d, J = 7.6 Hz, 2H), 4.42 (s, 2H), 3.82 (s, 3H); GCMS (R_t =14.35 min) calculated for $C_{18}H_{10}O_2$ 258.3, found 258.2.

Compound 41: The solvent was removed *in vacuo* to give 41 as a solid isolated as the TFA salt (8 mg, 0.032 mmol, 46%). ^{1}H NMR (400 MHz; CD_3OD): δ 3.97 (s, 2H), 3.75 (s, 2H), 2.70 (s, 2H); GCMS (R_t =7.03 min) calculated for C_8H_7NO 133.2, found 133.2.

Compound 42: The solvent was removed *in vacuo* to give 42 as a solid isolated as the TFA salt (9 mg, 0.032 mmol, 46%). ^{1}H NMR (400 MHz; CD_3OD): δ 4.00 (s, 2H), 3.58 (s, 2H), 2.95 (s, 2H); GCMS (R_t =8.43 min) calculated for $C_{10}H_7NO$ 157.2, found 157.2.

Compound 43: The solvent was removed *in vacuo* to give 43 as a solid isolated as the TFA salt (10 mg, 0.034 mmol, 48%). ^{1}H NMR (400 MHz; CD_3OD): δ 4.00 (s, 2H), 3.55 (s, 2H), 2.98 (s, 2H); GCMS (R_t =9.15 min) calculated for $C_{12}H_7NO$ 181.2, found 181.9.

Compound 44: The solvent was removed *in vacuo* to give 44 as a solid (8 mg, 0.044 mmol, 63%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.50 (t, J = 5.9, 2H), 7.38-7.28 (m, 3H), 4.45 (s, 2H), 1.94 (s, 1H); GCMS (R_t =15.06 min) calculated for $C_{13}H_8O$: 180.2, found 180.2.

Compound 45: The solvent was removed *in vacuo* to give 45 as a solid (9 mg, 0.044 mmol, 63%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.55 (t, J = 5.9, 2H), 7.40-7.30 (m, 3H), 4.45 (s, 2H), 1.94 (s, 1H); GCMS (R_t =10.08 min) calculated for $C_{15}H_8O$: 204.2, found 204.2.

Compound 46: The solvent was removed *in vacuo* to give 46 as a solid (9 mg, 0.039 mmol, 56%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.56 (t, J = 5.9, 2H), 7.44-7.38 (m, 3H), 4.45 (s, 2H), 1.94 (s, 1H); GCMS (R_t = 9.90 min) calculated for $C_{17}H_8O$: 228.3, found 228.3.

Compound 47: The solvent was removed *in vacuo* to give 47 as a solid (6 mg, 0.037 mmol, 53%) ^{1}H NMR (400 MHz; $CDCl_3$): δ 4.32 (s, 2H), 2.30 (t, J = 6.8 Hz, 3H), 1.55 (m, 2H), 1.42 (sextet, J = 7.2 Hz, 2H), 0.93 (t, J = 7.23 Hz, 3H); GCMS (R_t = 10.61 min) calculated for $C_{11}H_{12}O$ 160.2, found 160.2.

Compound 48: The solvent was removed *in vacuo* to give 48 as a solid (5 mg, 0.027 mmol, 38%) ^{1}H NMR (400 MHz; $CDCl_3$): δ 4.36 (s, 2H), 2.30 (t, J = 6.8 Hz, 3H), 1.55 (m, 2H), 1.42 (sextet, J = 7.2 Hz, 2H), 1.00 (t, J = 7.23 Hz, 3H); GCMS (R_t = 10.28 min) calculated for C13H12O 184.2, found 184.2.

Compound 49: The solvent was removed *in vacuo* to give 49 as a solid (9 mg, 0.028 mmol, 41%) ^{1}H NMR (400 MHz; $CDCl_3$): δ 4.38 (s, 2H), 2.35 (t, J = 6.8 Hz, 3H), 1.60 (m, 2H), 1.42 (sextet, J = 7.2 Hz, 2H), 1.02 (t, J = 7.23 Hz, 3H); GCMS (R_t = 11.71 min) calculated for $C_{15}H_{12}O$ 208.3, found 208.3.

Compound 50: The solvent was removed in vacuo to give compound 50 as a white solid (4 mg, 0.026 mmol, 82%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 4.36 (s, 4H); GCMS calculated for $C_8H_6O_2$ 134.0, found 134.1.

Compound 51: The solvent was removed in vacuo to give compound 51 as a white solid (3 mg, 0.018 mmol, 57%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 4.38 (s, 4H); GCMS calculated for $C_{10}H_6O_2$ 158.0, found 158.0.

Compound 52: The solvent was removed in vacuo to give compound 52 as a white solid (3 mg, 0.016 mmol, 49%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 4.35 (s, 4H); GCMS calculated for $C_{12}H_6O_2$ 182.0, found 182.1.

Compound 53: The solvent was removed in vacuo to give compound 53 as a solid (2 mg,

0.011 mmol, 34%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.01 (d, *J* = 7.5 Hz, 1H), 7.65 (t, *J* = 7.4 Hz, 1H), 7.32 (t, *J* = 7.5 Hz, 1H), 6.91 (d, *J* = 7.4 Hz, 1H), 4.38 (s, 2H). GCMS calculated for $C_{12}H_7NO$ 181.1, found 181.2.

Compound 54: The solvent was removed in vacuo to give compound 54 as a solid (2 mg, 0.009 mmol, 30%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.01 (d, *J* = 7.5 Hz, 1H), 7.65 (t, *J* = 7.4 Hz, 1H), 7.32 (t, *J* = 7.5 Hz, 1H), 6.91 (d, *J* = 7.4 Hz, 1H), 4.38 (s, 2H). GCMS calculated for $C_{14}H_7NO$ 205.1, found 205.1.

Compound 55: The solvent was removed *in vacuo* to give compound 55 as a solid (2 mg, 0.008 mmol, 27%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.01 (d, *J* = 7.5 Hz, 1H), 7.65 (t, *J* = 7.4 Hz, 1H), 7.32 (t, *J* = 7.5 Hz, 1H), 6.91 (d, *J* = 7.4 Hz, 1H), 4.38 (s, 2H). GCMS calculated for $C_{16}H_7NO$ 229.1, found 229.2.

Compound 56: The solvent was removed *in vacuo* to give 56 as a solid. (6 mg, 0.024 mmol, 56%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.55 (d, J=7.4 Hz, 2H), 7.50 (d, J=7.4 Hz, 2H), 7.38 (d, J= 7.4 Hz, 2H), 6.89 (d, J= 7.4 Hz, 2H), 4.75 (s, 2H), 3.83 (s, 3H); GCMS (R_t =16.89 min) calculated for $C_{20}H_{14}O_2$ 286.3, found 286.2.

Compound 57: The solvent was removed *in vacuo* to give 57 as a solid. (7 mg, 0.022 mmol, 52%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.98 (d, J=7.4 Hz, 2H), 7.58 (d, J=7.4 Hz, 2H), 7.50 (d, J=7.4 Hz, 2H), 6.95 (d, J=7.4 Hz, 2H), 4.75 (s, 2H), 3.85 (s, 3H); GCMS (R_t =13.38 min) calculated for $C_{22}H_{14}O_2$ 310.4, found 310.3.

Compound 58: The solvent was removed *in vacuo* to give 58 as a solid. (8 mg, 0.023 mmol, 55%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.80 (d, J=7.5 Hz, 2H), 7.50 (d, J=7.5 Hz, 2H), 7.45 (d, J=7.5 Hz, 2H), 6.85 (d, J=7.5 Hz, 2H), 3.80 (s, 2H), 2.85 (s, 3H); GCMS (R_t =11.43 min) calculated for $C_{24}H_{14}O_2$ 334.4, found 334.3.

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Chapter 6-Solid-Supported Glaser-Hay Couplings in the Microwave

I. Introduction

Microwaves are a component of the electromagnetic spectrum with wavelengths between 1 mm to 1 m, which corresponds to frequencies that vary from 0.3 and 300 GHz. This form of radiation is located between infrared radiation and radio waves on the electromagnetic spectrum.[9] (Figure 6.1)

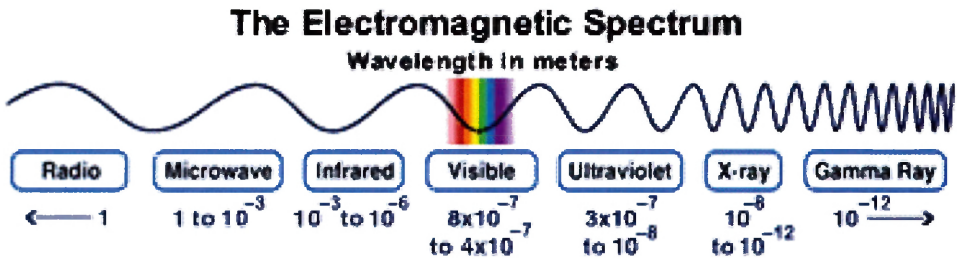


Figure 6.1: Electromagnetic Spectrum.[7]

In the past 30 years, microwaves have found widespread use in organic chemistry. Traditionally, heated organic reactions are performed on a hot plate, in a heating jacket, or in an oil bath. Unfortunately, these methods rely on convection and do not allow heat energy to transfer evenly throughout the reaction. These methods can also be slow to heat and can require consistent maintenance to regulate. To alleviate these temperature issues, microwave radiation has proven to be advantageous. Microwave technology has

also shown advantages such as the ability to significantly decrease reaction times and increase product yields.

Microwave radiation couples rotational energy to molecules in a reaction in order to produce efficient and thorough internal reaction heating. The two main components of microwave radiation involve an electric field and a magnetic field and can be referred to as dielectric heating.[13] In order for a reaction to increase in temperature when introduced to microwave radiation, the molecules present must have a dipole moment. The dipole will align with the electric field component and rotate to generate heat on a molecular level.[9] (Figure 6.2)

Figure 6.2: When introduced to an oscillating electric field, the dipoles of the molecules in the reaction will align and expend energy in order to conduct heat for the reaction.[9]

In order for the dipole of the molecules involved in the reaction to directly interact with the oscillating electric field, the solvent used for a microwave reaction should lack a permanent dipole moment of its own, rendering it transparent to the microwave irradiation. Good solvents (that have low dielectric constants) for use with microwave synthesis include ethyl acetate, benzene, and tetrahydrofuran. Similarly, by using

microwave transparent vessels such as a borosilicate glass vessel these mechanisms can combine to provide a consistent heating of the reaction.[14] The advantage of using microwave radiation to evenly heat a reaction is illustrated in Figure 6.3.

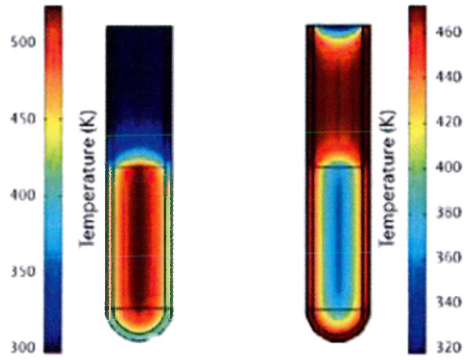


Figure 6.3: When a reaction vessel is heated through microwave radiation in the proper instrument (left), the heat is distributed evenly throughout the solution. However, when heated by a conventional oil bath (right), the reaction vessel is heated first, causing heat to be distributed improperly and the solution to remain at a lower temperature.[4]

Initially, scientific microwaves were found to be of great importance to the medicinal and combinatorial chemistry industries. They then were transitioned into great use in the fields of synthesis and organic chemistry, and most recent, have been found helpful in the developing area of green chemistry.[3] They were first introduced into organic chemistry in 1986 through a study of multiple reactions involving different solvents.[1] It was discovered that reaction rates could be rapidly decreased through the use of the microwaves. The first researchers focused on using microwave irradiation to study and decrease reaction times of Diels-Alder, Claisen, and –ene reactions.[2] After this initial

report, microwaves began to find widespread use throughout organic chemistry, affording increased product yields and shorter reaction times.

The first scientific research performed with microwave technology used commercial microwave ovens that can be typically find in a kitchen. However, these were found to be unsafe, and would sometimes cause explosions.[9] Domestic microwaves also did not possess the ability to consistently control time and temperature, and did not provide a specific chamber for the reaction that allowed for the application of pressure to the reaction. Moreover, the microwaves themselves were unable to be focused on the reaction, and thus did not confer an efficient mechanism for heating. Therefore, a scientific microwave was introduced into the industry that would allow for a vessel to be subjected to a focused beam of microwave irradiation, and a reaction to occur with consistent power, temperature, and pressure settings.[15] (Figure 6.4)

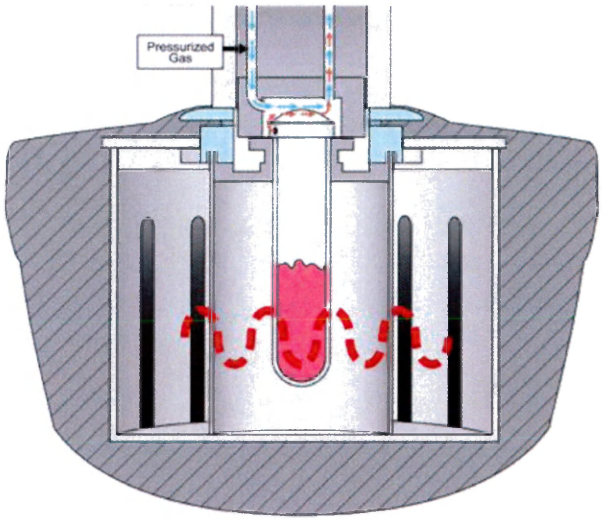


Figure 6.4: Diagram demonstrating a CEM Microwave and its ability to hold a transparent reaction vessel and allow an even, pressurized distribution of microwave radiation.[17]

Due to the increased availability of scientific microwaves, as well as an improved knowledge of their uses and function, microwaves are becoming a common tool in modern organic chemistry. To date, numerous reactions have been shown to occur at an accelerated reaction rate in a microwave. Leadbetter et al. report performing a microwave-mediated Suzuki coupling of boronic acids and aryl halides in only 5-10 minutes.[10] Heck reactions, typically involving alkenes and organohalides, were reported to occur in the microwave in under 5 minutes, compared to a normal multiple hour reaction time.[16] Of recent importance is the application of microwave chemistry to the popular Click reactions that are finding widespread use throughout the areas of biochemistry, medicinal chemistry, and organic chemistry.[11] Microwave-mediated

synthesis has also been expanded to Diels-Alder reactions, cycloadditions, oxidations, Mitsunobu reactions, and glycosylations.[8]

With a significant amount of successful microwave-mediated reactions presented in literature, we aimed to transition the previously reported solid-supported Glaser-Hay reactions into the microwave. In doing so, the potential to significantly reduce the reaction time of the Glaser-Hay reactions from 16 hours to minutes represented a substantial enhancement to the methodology.

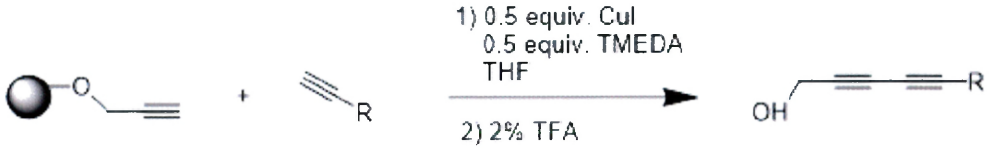
II. Results and Discussion

A. Development of Glaser-Hay Reaction Conditions Utilizing the Microwave

With previously optimized solid-supported Glaser-Hay reaction conditions (see Chapter 5), efforts were made to transition the reactions into the microwave and create a small library of asymmetrical diynes. By utilizing microwave-mediated synthesis, the goal was to drastically reduce reaction times, while still synthesizing the asymmetric diyne in good yield. Initial microwave attempts were performed with a resin immobilized with propargyl alcohol and phenylacetylene was employed as the soluble alkyne. Phenylacetylene was initially selected for optimization due to its high microwave reactivity and ability to easily track the reaction via TLC analysis. THF remained a viable solvent choice due to its microwave transparency.

To begin, the reactions were performed using the described conditions optimized previously. (Scheme 6.1)

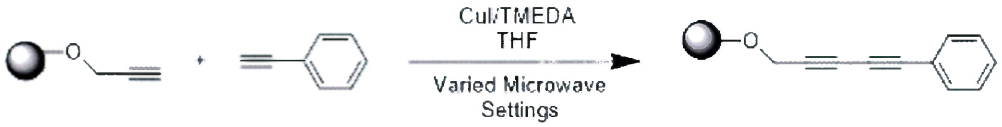
Scheme 6.1



It was believed that by irradiating the reactions with specific microwave powers the reactions would be driven to completion in a matter of minutes. However, 10-minute reaction attempts at constant power settings of 100 W, 200 W, or 300 W, showed little to no product formation. It was discovered that while irradiating with the constant power, the temperature of the microwave vessel would rise between 140˚C and 180˚C. This high temperature was hypothesized to be potentially degrading the resin, forming little product. Moving forward, conditions were altered using a constant temperature setting in order to control the heat and ideally prevent resin degradation.

Gratifyingly, initial attempts with the controlled temperature setting began to yield diyne product. Numerous reaction times at varied temperatures were examined with the phenylacetylene and propargyl alcohol immobilized resin in order to find the optimal conditions producing the highest yield. (Table 6.1)

Table 6.1: Tested and varied microwave conditions.



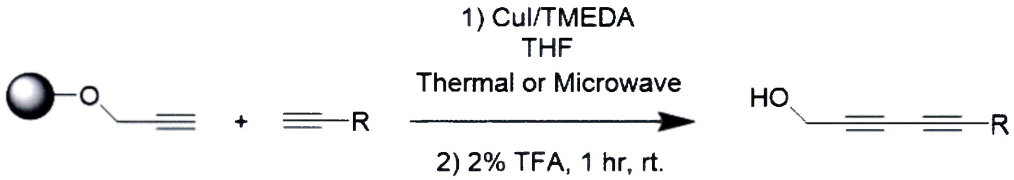
Temperature	Time	%Yield
40 °C	20:00 min.	No Product
60 °C	05:00 min.	26%
60 °C	10:00 min.	77%
80 °C	05:00 min.	No Product
80 °C	10:00 min.	77%
100 °C	05:00 min.	No Product
100 °C	10:00 min.	63%

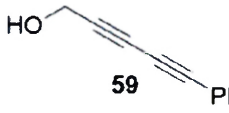
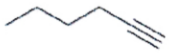
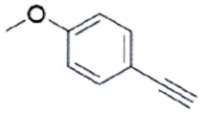
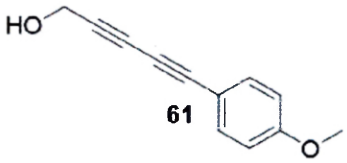
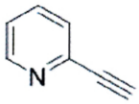
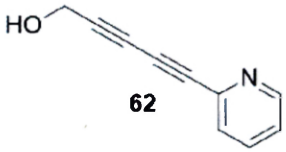
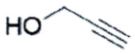
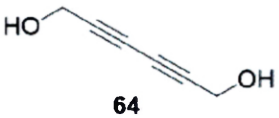
As both the 60 °C for 10:00 minutes and the 80 °C for 10:00 minutes reactions produced the same yield, the optimum and best conditions for the reaction were believed to be at 60 °C for 10:00 minutes. This demonstrated that the overall reaction time could be drastically reduced from the previously established time of 16 hours to a mere 10 minutes, which would allow for rapid preparation of asymmetrical diynes. Moving forward, it was believed that these conditions could be applied to a number of terminal alkynes in order to prepare a small library of solid-supported Glaser-Hay microwave reactions.

B. Preparation of a Microwave Synthesized Diyne Library

Continued efforts were made to analyze the methodology via the synthesis of additional asymmetrical diynes using microwave irradiation. As elucidated with the propargyl alcohol resin and phenylacetylene coupling, it was believed that reactions could occur at 60 ˚C for 10:00 minutes. The propargyl alcohol resin was utilized with the following terminal alkynes: propagyl alcohol, ethynylanisole, hexyne, propargyl amine, and ethynylpyridine. Upon completion of microwave irradiation, reactions were transferred to a filtered syringe and washed 5 x with DCM and MeOH to remove catalyst and unreacted alkyne. The product was then cleaved from the resin with 2% trifluoroacetic acid in DCM, the product was filtered into a dry vial, and the solvent was removed *in vacuo*. To analyze the reaction, each product was examined by ^{1}H NMR. Upon initial reaction attempts at 60˚C for 10:00 minutes with each soluble alkyne, it was found that some reactions did not afford any product. Moving forward, reaction conditions were altered to yield product with the varied alkynes to elucidate a general microwave protocol. Temperatures between 60˚C and 120˚C and reaction times from 10:00 minutes to 20:00 minutes were examined. The reactions were then analyzed via ^{1}H NMR and yields were determined. Optimum reaction conditions and the produced yields when reacted with the propargyl alcohol loaded resin can be found in Table 6.2.

Table 6.2



Alkyne	Product	% Yield Thermal	% Yield Microwave
	 **59**	**95%**	**77% ***
	 **60**	**84%**	**67%**
	 **61**	**99%**	**75%**
	 **62**	**55%**	**90%**
	 **63**	**40%**	**92%**
	 **64**	**99%**	**90%**

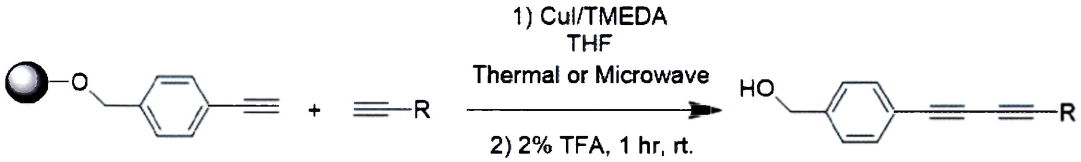
***Can be performed at 60˚C for 10:00.**

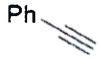
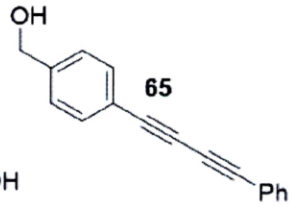
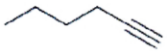
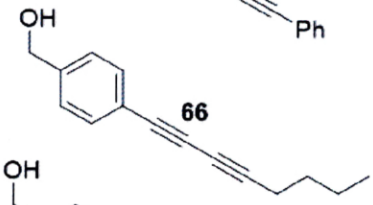
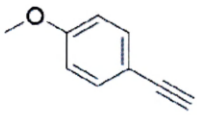
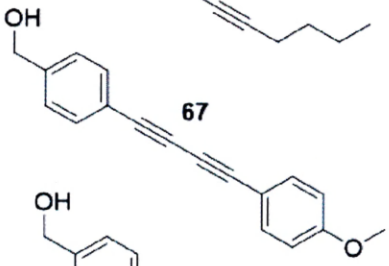
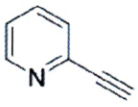
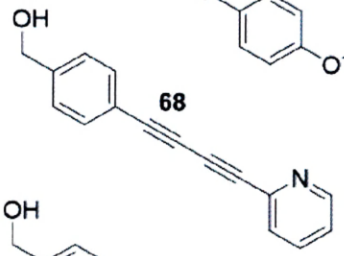
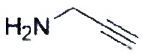
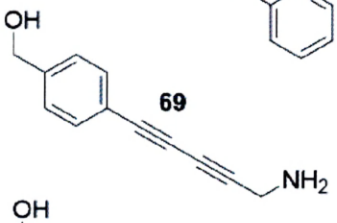
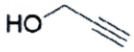
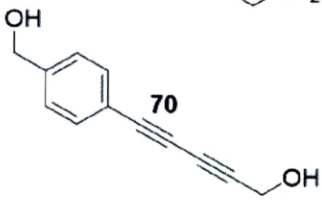
Using the propargyl alcohol immobilized resin, product formation was seen with temperature settings of 80° for 20 minutes. Products were obtained in good purity and needed no further purification. Based on yields, the alkyne reactants including a nitrogen atom reacted better under microwave conditions than under standard thermal conditions. This may be due to the fact that nitrogen containing compounds are very microwave reactive, as they exhibit a strong dipole moment, increasing their interactions with the microwave irradiation. Moreover, we hypothesize that due to the quick rate of reaction in the microwave, no catalyst poisoning occurs from soluble alkyne coordination with the copper as seen in the original methodology (See Chapter 5). Ultimately, this indicates that the microwave will be a useful tool for Glaser-Hay reactions involving basic functionalities. The compound containing the hexyne moiety exhibits the lowest yield of the six soluble alkynes, perhaps due to the fact that an alkyl side chain is not very microwave reactive. Overall, we determined that the utilization of microwave technology with the solid-supported Glaser-Hay methodology permitted synthesis of a small library of asymmetric diynes in minutes as opposed to the standard 16 hour reaction time.

C. Investigations with Alternative Immobilized Alkynes

In order to expand the scope of this methodology, a trityl chloride resin was loaded with 4-ethylnyl benzyl alcohol. This immobilized resin was then exposed to the same alkynes and similar microwave conditions to generate a small library of diynes created with the 4-ethynyl benzyl alcohol resin. Results can be seen in Table 6.3

Table 6.3



Alkyne	Product	% Yield Thermal	% Yield Microwave
	 **65**	**96%**	**78%***
	 **66**	**98%**	**76%**
	 **67**	**73%**	**76%**
	 **68**	**53%**	**86%**
	 **69**	**96%**	**86%**
	 **70**	**40%**	**86%**

***Can be performed at 80˚C for 20:00.**

Initial reaction attempts afforded a mix of starting material and desired asymmetrical product. This can be attributed to the fact that the 4-EBA functionality is highly adsorbing of microwave irradiation, heating much more rapidly than the immobilized propargyl alcohol resin. Overall, this resulted in very low microwave input into the reactions. Because of this, the 4EBA resin required higher temperatures, as well as microwave pulses to drive the reaction to completion.

To alleviate the mixture of starting material and product, 2:00 minute reaction pulses at 120° were used to total a 20:00 minute reaction time, allowing the reaction to return to room temperature between the pulses. By pulsing the reaction, the microwave power was kept higher and came in bursts rather than one initial ramp of power that decreases overtime. These pulses were shown to drive most reactions to completion and higher yields were obtained. However, reaction with hexyne was still not driven to completion and a mix of cleaved 4-ethynylbenzyl alcohol and desired product was could be seen on ^{1}H NMR. Therefore, a column was used to obtain the desired asymmetrical 4EBA-hexyne product in low yield. This follows the pattern shown previously where the hexyne yield on the propargyl alcohol immobilized resin was also one of the lowest. The alkyl side-chain is microwave transparent and therefore produces a low yield of desired product. However, other alkynes possessing more reactive species were shown to react better with irradiation and produce asymmetrical products in good yields using the microwave vessel. This was especially the case in regards to the pyridine and amino

functional groups, which both produced good yields due to their good microwave irradiation absorption properties and increased microwave reactivity.

Overall, it can has been demonstrated that solid-supported Glaser-Hay reactions can take place in the microwave using mild reaction conditions. Further research could be performed in order to produce even higher yields and eliminate unwanted side products from various immobilized resins. Moreover, more complex soluble and immobilized alkynes can be explored to further probe the limits of the microwave mediated reaction.

III. Materials and Methods

General. Solvents and reagents were obtained from either Sigma-Aldrich or Fisher Scientific and used without further purification, unless noted. Tritylchloride resin, 100-200 mesh, 1% DVB crosslinking, was purchased from Advanced Chemtech. Microwave reactions took place in a CEM Discover System, model no. 908005. Reactions were conducted under ambient atmosphere with non-distilled solvents. NMR data were acquired on a Varian Gemini 400 MHz.

Immobilization of Alcohol onto Trityl Chloride Resin in Low Loading Conditions

Trityl chloride resin (200 mg, 0.36 mmol, 1 equiv.) and dichloromethane (5 mL) were added to a flame dried vialδ. The resin was swelled at room temperature with gentle stirring for 15 min. Desired alcohol (~1.2 equiv.) was added to reaction, followed by triethylamine (10.0 μL, 0.072 mmol, 0.2 equiv). The mixture was stirred at room temperature for 16 h. The resin was transferred to a syringe filter and washed with DCM

and MeOH (5 alternating rinses with 5 mL each). The resin was swelled in CH_2Cl_2 and dried under vacuum for 45 min before further use.

Sample of Glaser Hay Coupling Protocol at 60 ˚C

Soluble alkyne (0.70 mmol, 10 equiv.) was added to a flame dried microwave vial containing the propargyl alcohol derivatized trityl resin (100 mg, 0.07 mmol, 1 equiv.), and tetrahydrofuran (2.0 mL). The copper catalyst (10 mg, 0.053 mmol.) and tetramethylethylenediamine (50 µL.) were added to a separate flame-dried vial then dissolved in tetrahydrofuran (2.0 mL). The catalyst mixture was then added to the resin in one portion and placed in the microwave reactor to run for allotted time at specific temperature. The completed reaction was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each). The product was then cleaved from the resin by treatment with 2% TFA (DCM, 1 h) and filtered into a vial. Product was analyzed via ^1H NMR.

Analytical Data

Compound 59: The solvent was removed *in vacuo* to give compound 59 as a solid (6 mg, 0.039 mmol, 77%). ^1H NMR (400 MHz; $CDCl_3$): δ 7.49 (t, J = 5.9, 2H), 7.34-7.26 (m, 3H), 4.45 (s, 2H), 1.94 (s, 1H); GCMS (R_t=9.20 min) calculated for $C_{11}H_8O$ 156.1, found 156.2.

Compound 60: The solvent was removed *in vacuo* to give compound 60 as a solid (5 mg, 0.034 mmol, 67%) ^1H NMR (400 MHz; $CDCl_3$): δ 4.32 (s, 2H), 2.29 (t, J = 6.8 Hz, 3H), 1.52 (m, 2H), 1.42 (sextet, J = 7.2 Hz, 2H), 0.91 (t, J = 7.23 Hz, 3H); GCMS (R_t=7.29 min) calculated for $C_9H_{12}O$ 136.1, found 136.0.

Compound 61: The solvent was removed *in vacuo* to give compound 61 as a solid (7 mg, 0.038 mmol, 75%) ^1H NMR (400 MHz; $CDCl_3$): δ 7.44 (d, J = 7.6 Hz, 2H), 6.85 (d, J = 7.6, 2H), 4.14 (s, 2H), 3.82 (s, 3H); GCMS (R_t=9.93 min) calculated for $C_{12}H_{10}O_2$ 186.1, found 186.2.

Compound 62: The solvent was removed *in vacuo* to give compound 62 as a solid (7 mg, 0.045 mmol, 90%). ^1H NMR (400 MHz; $CDCl_3$): δ 8.01 (d, J = 7.5 Hz, 1H), 7.65 (t, J = 7.4 Hz,

1H), 7.32 (t, J = 7.5 Hz, 1H), 6.91 (d, J = 7.4 Hz, 1H), 4.38 (s, 2H). GCMS (R_t=8.77 min) calculated for $C_{10}H_7NO$ 157.1, found 157.2.

Compound 63: The solvent was removed *in vacuo* to give compound 63 as a solid (5 mg, 0.046 mmol, 92%). Using 7 as a starting material (3.5 mg, 0.032mmol, 91%). ^{1}H NMR (400 MHz; CD_3OD): δ 5.21 (s, 2H), 4.35 (s, 2H), 3.39 (s, 2H); GCMS (R_t=6.01 min) calculated for C_6H_7NO 109.1, found 109.1.

Compound 64: The solvent was removed *in vacuo* to give compound 64 as a solid (5 mg, 0.045 mmol, 90%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 4.36 (s, 4H); GCMS (R_t=7.88 min) calculated for $C_6H_6O_2$ 110.0, found 110.1.

Compound 65: The solvent was removed *in vacuo* to give compound 65 as a solid. (9 mg, 0.039 mmol, 78%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.53 (d, J=7.5 Hz, 4H), 7.45 (t, J=7.5 Hz, 4H), 7.30 (m, J=7.5, 5H), 4.74 (s, 2H); GCMS (R_t =10.75 min) calculated for $C_{17}H_{12}O_2$ 232.1, found 232.1.

Compound 66: The solvent was removed *in vacuo* to give compound 66 as a solid. (4 mg, 0.019 mmol, 38%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.52 (d, J=7.5 Hz, 2H), 7.31 (d, J=7.4 Hz, 2H), 4.73 (s, 2H), 2.32 (t, J=7.1 Hz, 2H), 1.56 (quintet, J=7.1 Hz, 2H), 1.42 (m, 2H), 0.95 (t, J=7.1 Hz, 3H); GCMS (R_t =9.85 min) calculated for $C_{15}H_{16}O$ 212.1, found 212.2.

Compound 67: The solvent was removed *in vacuo* to give compound 67 as a solid. (10 mg, 0.038 mmol, 76%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.53 (d, J=7.5 Hz, 2H), 7.30 (d, J=7.5 Hz, 2H), 7.40 (d, J=7.5 Hz, 2H), 6.95 (d, J=7.5 Hz, 2H), 4.65 (s, 2H), 3.96 (s, 3H); GCMS (R_t =13.10 min) calculated for $C_{18}H_{14}O_2$ 262.1, found 262.1.

Compound 68: The solvent was removed *in vacuo* to give compound 68 as a solid. (10 mg, 0.043 mmol, 86%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.61 (t, J=8.6 Hz, 1H), 7.55 (t, J=8.6 Hz, 1H), 7.53 (d, J=7.3 Hz, 2H), 7.40 (d, J=7.4 Hz, 1H), 7.31 (d, J=7.3 Hz, 2H), 7.25 (s, 1H), 4.65 (s, 2H); GCMS (R_t =10.75 min) calculated for $C_6H_{11}NO$ 233.1, found 233.3

Compound 69: The solvent was removed *in vacuo* to give compound 69 as a solid. (8 mg, 0.043 mmol, 86%). ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.40 (d, J=7.5 Hz, 2H), 7.33 (d, J=7.5 Hz, 2H), 4.61 (s, 2H), 3.28 (s, 2H). GCMS (R_t=9.92 min) calculated for $C_{12}H_{11}NO$ 185.1, found 185.2.

Compound 70: The solvent was removed *in vacuo* to give compound 70 as a solid (8 mg, 0.043 mmol, 86%). ^{1}H NMR (400 MHz; $CDCl_3$): ∂ 7.49 (d, J = 8.0 Hz, 2H), 7.44 (d, J = 8.0 Hz, 2H), 4.72 (s, 2H), 4.35 (s, 2H); GCMS (R_t=9.94 min) calculated for $C_{12}H_{10}O_2$ 186.1, found 186.1.

Acknowledgments

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Chapter 7- Glaser-Hay Bioconjugation

I. Introduction

A. An Introduction to Bioconjugation

The term bioconjugation denotes a reaction that links together two molecules, typically with at least one of the two being of biological origin or relevance. (Figure 7.1) Many bioconjugation methods involve the incorporation of biological probes, antibodies, or enzymes. The bioconjugation is also often characterized by the type of linkage it produces between the two molecules, be it a covalent or non-covalent interaction.

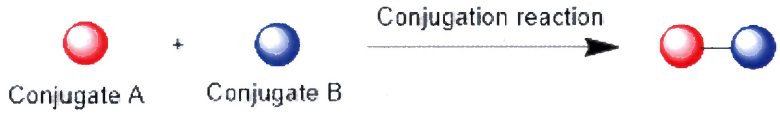


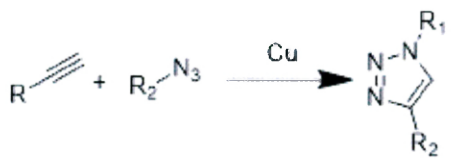
Figure 7.1: Standard depiction of molecular conjugation.

Bioconjugation techniques have proven to be an effective strategy for studying numerous biological process and have also paved the way for drug and therapeutic discovery. Often they are utilized in many industries including medical, diagnostic, life science, and materials science.[1] For example, a clinical chemistry study reported the ability to conjugate intelligent polymers to protein bodies. Intelligent polymers are polymeric molecules that respond to small changes in their environment such as temperature, pH, or light with changes in their physical state or properties. Incorporation of these as bioconjugates allows for the direct study of the effects of changes in the

protein environment leading to further studies in diagnostics as a result of the polymer response to environmental stimuli.[3]

Numerous bioconjugations involving protein and a conjugate are well known, including streptavidin-enzyme, antibody-enzyme, and antibody-drug conjugates. There are, however, limitations to bioconjugate formation. One major limitation is the site-specific attachment of the desired conjugate. For example, to an antibody containing numerous lysine sites, the researcher does not always have complete control over which site the conjugate will attach, leading to a potential loss of function of the antibody. Therefore, it would be beneficial to site-specifically control the attachment site for bioconjugates, in order to ensure the proper function of the developed system.[1] A well-known strategy of site-specific bionconjugation involves the use of unnatural amino acids in order to introduce a functional group to participate in a selective reaction. Recent advances in protein alteration and bioconjugation have begun to introduce functionalities that may undergo photocrosslinking, alkyne coupling reactions, oxime formations, and 1,3 dipolar cycloadditions.[1] The 1,3 dipolar cycloadditions were first introduced by Rolf Huisgen in 1961 and have proven very popular among many researchers.[4] These reactions take place between an azide and an alkyne functional group (either terminal or internal) to give rise to a stable, covalent triazole linker.[5] (Scheme 7.1)

Scheme 7.1



Although this reaction has found a wide variety of uses in the area of "click" chemistry, it is still necessary to pursue other reactions that may be ideal candidates for bioconjugations. There are a variety of other reactive moieties present within already developed UAAs that could be efficiently utilized for bioconjugations, and the pursuit of a wider range of reaction possibilities is exciting in the area of bioconjugate chemistry.

As mentioned, unnatural amino acids have promising uses in the area of bioconjugation. Figure 7.2 shows a variety of known phenylalanine derivatives that involve unique functional groups that can undergo potential bioconjugation reactions. A halogenated phenylalanine (7.2.A) may be reacted in numerous ways. For example, a boronic ester can be introduced to afford a Suzuki coupling reaction.[9] A halogenated species can also be used for a Heck reaction when introduced to an alkene functional group[7], or for Songoshira coupling when reacted with a terminal alkyne.[8] Phenylalanine derivatives containg azide (7.2.B) or terminal alkynes (7.2.C) can undergo the popular Huisgen reaction[5], and acetyl groups (7.2.D) on phenylalanine can be used to form oxime linkages.[10]

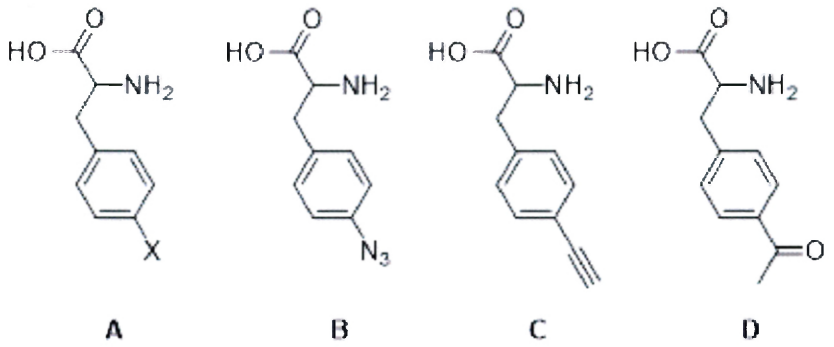


Figure 7.2: Various phenylalanine derivatives that may be incorporated into proteins in order to undergo a variety of reactions. Introducing the reactive functional groups allows for many possible bioconjugation methods.

In order to ensure an effective bioconjugation reaction, the reaction technique should include mild aqueous reaction conditions. The reaction should also be able to produce a stable bond between reactants with a high degree of chemoselectivity and bioorthogonality.[11]

B. Benefits of Using Glaser-Hay Coupling for Bioconjugation

Due to the recent optimization of the Glaser-Hay reaction for solid-supported synthesis, it was envisioned that this reaction could also be employed for bioconjugations. The Glaser-Hay reaction involves the coupling of two terminal alkynes to produce a linear moiety that could give rise to further reactions or biological bioconjugate additions. It is an optimal reaction for bioconjugation due to the formation of the very stable covalent carbon-carbon bond under mild reaction conditions. Additionally, it provides a linear,

highly oxidized product that could be further reacted using numerous known reactions or used for additional biological conjugate reactions. This reaction also tolerates a wide range of functional groups, and therefore a plethora of reaction partners containing alkyne functional groups could potentially be employed and possibly give rise to new therapeutic agents.

II. Results and Discussion

A. Utilizing the Glaser-Hay Reaction for Protein Bioconjugation

Before employing the Glaser-Hay reaction toward bioconjugations, the reaction had to be translated to aqueous conditions. Upon a thorough literature search, we could not find evidence that the Glaser-Hay reaction had been previously performed in an aqueous solution. Therefore, a proof-of-concept Glaser-Hay reaction was conducted in water utilizing phenylacetylene to afford a homodimer. It was hoped that the previously developed solid-supported Glaser-Hay methodology (see chapter 5) would easily translate to aqueous conditions using the same protocol. To start, phenylacetylene was added to a vial along with the copper catalyst system consisting of CuI and TMEDA. In place of THF, water was used as a solvent for both the alkyne and the copper catalyst system. This reaction was then stirred at room temperature for 16 hours. Upon reaction completion, the product was extracted using EtOAc and H_2O, and solvent removed *in vacuo*. Gratifyingly, homodimer product was obtained in good yield (96%) without further purification, proving that the Glaser-Hay reaction could be performed in an aqueous,

biological setting. Due to the success of the aqueous conditions, it was believed that the Glaser-Hay coupling would be effective in the aqueous conditions required for protein bioconjugations.

To apply the Glaser-Hay reaction to a protein bioconjugation, the protein must contain an alkyne functional group to act as a handle for reaction with a second alkyne functional group on the molecule designated for conjugation. In order to introduce the alkyne functional group, the approach using orthogonal tRNAs and aminoacyl-tRNA synthetases described in Chapter 1 was utilized to incorporate an alkynyl unnatural amino acid, propargyloxyphenylalanine (Figure 7.3), into a reporter protein. The synthesis of the UAA was performed based on literature conditions.[2] Conveniently, an aaRS has already been evolved to recognize the alkynyl UAA, and integrated into an expression plasmid known as pEVOL-*p*PrF.[2]

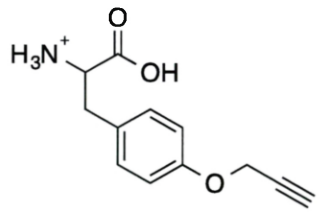


Figure 7.3: Propargymoxyphenylalanine UAA employed in bioconjugations.

GFP is an ideal reporter protein due to its high fluorescence activity, and was therefore an optimal choice for bioconjugation optimization. The GFP gene used in this research contains a TAG codon introduced at the surface exposed residue at codon 151

which allows for an accessible site for UAA expression. As well as being convenient for UAA expression, a surface exposed residue is important for bioconjugation. If the residue is buried within the protein, bioconjugation reactions would be much harder or impossible to perform.[6]

When the mutated mRNA transcript with the inserted TAG codon is introduced to the ribosome, protein translation takes place, installing the UAA when it is present in the expression media. However, when an unnatural amino acid is not present, the TAG codon terminates translation and full protein is not produced. Overall, it was believed that by inserting the alkynyl handle at this position, bioconjugation attempts utilizing the Glaser-Hay reaction could be investigated. (Figure 7.4)

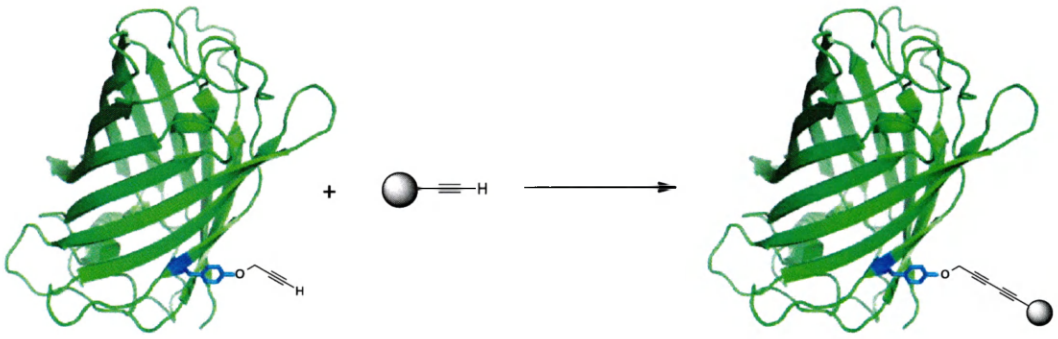


Figure 7.4: The structure of GPF consists of an outer beta-barrel and an internal chromophore.[13] This diagram shows GFP with alkynyl UAA insertion at the surface-exposed position 151 undergoing the envisioned Glaser-Hay reaction to produce the conjugated protein species.

BL-21(DE3) *E. coli* were co-transformed with the pEVOL-*p*PrF and pET-sfGFP-Y151TAG plasmids and the mutant GFP was expressed in both the presence and absence of the alkynyl UAA. Due to the TAG codon at position 151, when *p*PrF is not introduced to the protein, translation terminates which is indicated by absence of the GFP band in the SDS-PAGE gel. (Figure 7.7; Lanes 6 and 7) Lane 6 shows presence of the GFP-*p*PrF-151 mutant which permitted research of Glaser-Hay bioconjugation optimization to proceed.

Initial reaction attempts included the mutant GFP and a small molecule AlexaFluor-488 alkyne, which was a good marker for this reaction due to the ability to easily track bioconjugations through fluorescence. A 500 mM solution of CuI and a 500 mM solution of TMEDA in H_2O were prepared for use as the catalyst system. The CuI and TMEDA were reacted together for 10 minutes at 37 °C to allow equilibration and catalyst complex formation. The fluorophore was then added to the catalyst mixture, followed by the GFP mutant. Reactions were incubated at either 37 °C or 4 °C in order to determine optimal reaction temperatures. Initially, reactions were left overnight. However, once washed and analyzed by gel electrophoresis it was discovered that there was a lack of protein in the completed reactions, signifying protein degredation. Reaction times were therefore decreased to determine optimal time conditions. Overall, the longer reaction times showed a decrease in fluorescence and an absence of GFP. It was hypothesized that the copper in the catalyst system may be degrading protein throughout the longer time periods. To determine the best conditions for the highest yield of bionconjugated protein, time courses were performed at both 37 °C and 4 °C. Upon completion, reactions were analyzed via SDS-PAGE (Figure 7.5) and the ratio of GFP and reaction fluorescence was

determined and plotted. (Figure 7.6) Ultimately, it was determined that 6 hour reactions at 4 °C appeared to be optimal for the Glaser-Hay bioconjugation.

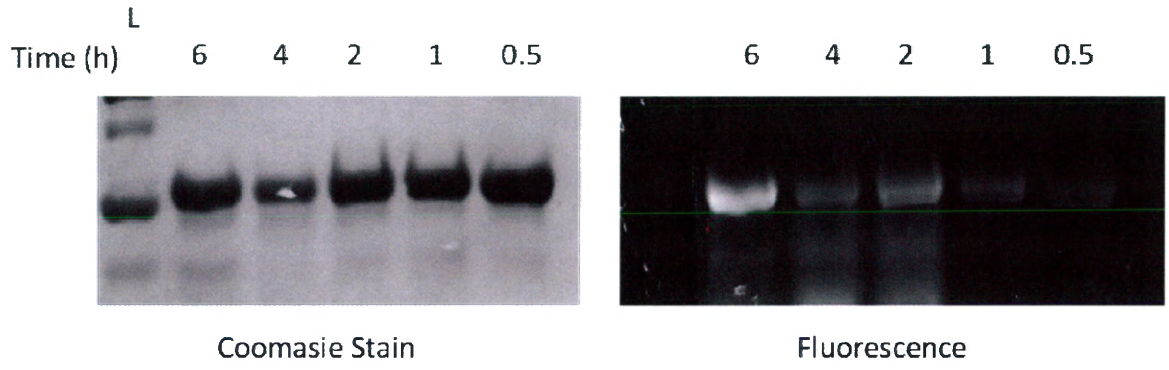


Figure 7.5: SDS-PAGE analysis of reactions completed between 0.5 and 6 hours, both pre-Coomassie Blue staining (for fluorescence) and post staining. The densitometry of each band was then measured to generate a ratio of total protein to fluorescently labeled protein.

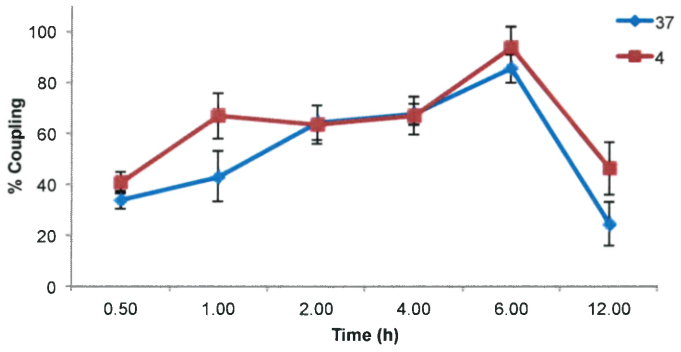


Figure 7.6: Glaser-Hay timecourse optimization. Ratio of intact GFP to fluorescence of SDS-PAGE gel bands. Error bars indicate standard deviation based on readings. Results indicate highest %coupling and optimum reaction conditions at 4 °C for 6 hr. Extended periods of time lead to protein degradation.

Upon determining optimal conditions, the reactions were performed using the mutant GFP and the same copper/TMEDA catalyst system and the AlexaFluor-488 alkyne. Upon reaction completion, any excess fluorophore was removed by centrifugal concentration and washed several times with PBS. Reaction products were then analyzed via SDS-PAGE and LC/MS.

The gel results demonstrated a successful Glaser-Hay bioconjugation through both initial fluorescent imaging combined with Coomassie Blue staining imaging. (Figure 7.7) As seen in Lane 2 and 4, with the presence of both the catalyst system and the alkyne fluorophore, fluorescent labeling of the GFP is apparent at both 37 °C and 4 °C. A control reaction was also performed that did not have the catalyst system introduced. Lane 6 shows that the control reaction still showed presence of the GFP, but the protein had no fluorophore fluorescence, thus confirming the Glaser-Hay coupling of the GFP to the alkyne fluorophore by using the copper catalyst system.

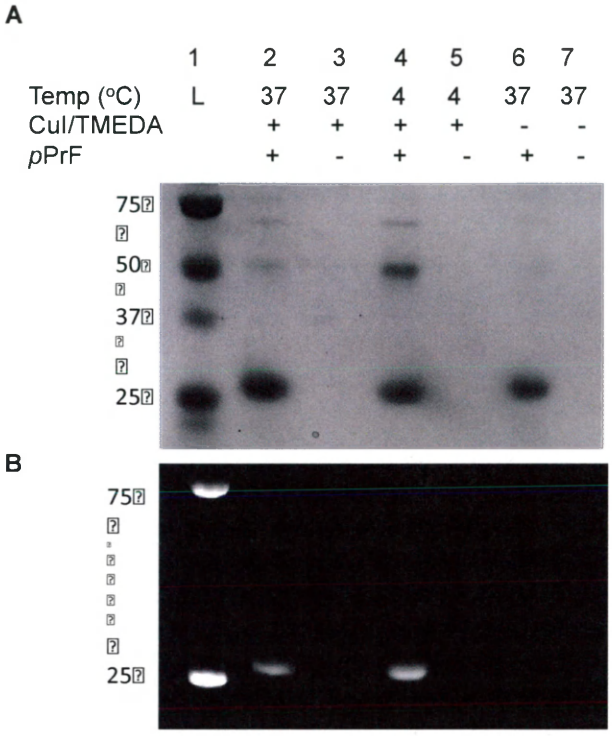


Figure 7.7: Glaser-Hay bioconjugation at both 37 °C and 4 °C. (A) SDS-PAGE analysis after Coomassie Blue staining showing the successful incorporation of *p*PrF into GFP (lane 6 and 7) due to presence of protein band in 6 and lack of in 7. Bands in 2 and 4 indicate presence of conjugated mutant protein upon reaction completion using Glaser-Hay conditions. (B) SDS-PAGE analysis indicating successful conjugation of protein to fluorophore. The presence of fluorescent bands in lanes 2 and 4 suggest successful coupling while lack of fluorescence in lanes 3 and 5 indicate no attached fluorophore.

Overall, this research was able to transition the Glaser-Hay reaction to an aqueous environment in order to afford a novel bioconjugation methodology. It was applied successfully to protein-fluorophore conjugations, and can be further advanced to include other molecules and conjugates. Due to the wide reaction scope, this method could be a

viable alternative to the popular "click" conjugations and could be further studied to optimize all benefits.

B. Toward Glaser-Hay Solid-Support Bioconjugations

To further enhance the scope of the Glaser-Hay bioconjugation method, research focus moved to utilizing this reaction for solid-support protein immobilizations. Initial efforts toward this focused on applying reaction conditions to a propargyl alcohol derivatized Sepharose resin[12] and the AlexaFluor fluorophore alkyne. A control reaction was conducted without the presence of the copper catalyst system to assess that there was not non-specific coupling to the resin. Previously established reaction conditions were employed. Upon reaction completion the resin was washed several times with aliquots of PBS to eliminate any excess fluorophore, and results were viewed by fluorescent imaging. (Figure 7.8) Fluorescence was only viewed in the reaction that had contained the copper catalyst, indicating success of the propargyl alcohol resin with the fluorophore.

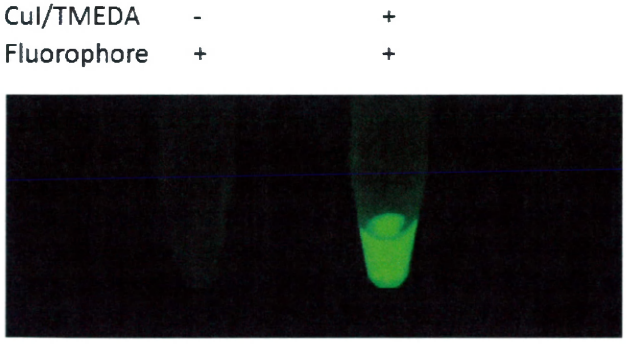


Figure 7.8: Propargyl alcohol immobilized Sepharose resin reacted with an alkynyl fluorophore.

Attempts were then made to translate this methodology to the coupling of protein with the immobilization resin. An immobilized protein could be employed for further reactions or as a biorthogonal handle for biological applications. The GFP-*p*PrF-151 mutant was again used alongside of either a propargyl alcohol or a hexynol immobilized Sepharose resin. Using the optimized Glaser-Hay conditions, the protein was reacted with the immobilized resin for 6 hours at 4 °C and upon completion was concentrated by centrifugation and washed five times with PBS to eliminate any excess or unreacted protein. The purified reactions were then viewed with fluorescent imaging. (Figures 7.9 and 7.10)

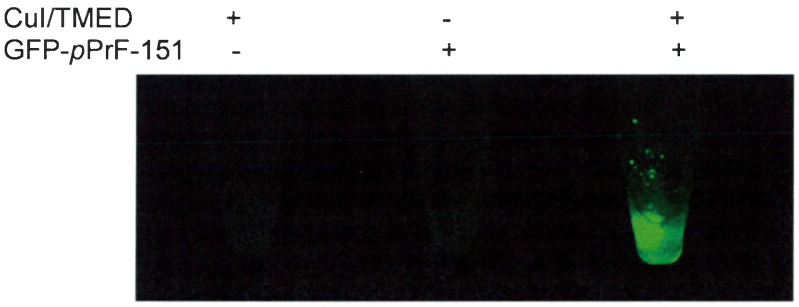


Figure 7.9: Reaction including the mutant protein and immobilized propargyl alcohol Sepharose resin. Strong fluorescence in the presence of both catalyst and GFP-*p*PrF-151 indicate a successful coupling. Lack of fluorescence in control reactions not containing protein or copper catalyst indicate no coupling.

Figure 7.9 depicts three reactions using the propargyl alcohol Sepharose resin. One control reaction was performed in the absence of protein and there is no fluorescence

observed after resin washing. Another control reaction was conducted with protein and resin but no copper catalyst system. Gratifyingly, there was also no fluorescence viewed upon reaction purification. However, in the reaction with the immobilized resin, GFP-*p*PrF-151, and CuI/TMEDA a bright fluorescence signal was observed indicating the successful coupling of the GFP mutant to the immobilized Sepharose resin. Figure 7.10 shows the same reaction and controls performed with a hexynol immobilized resin.

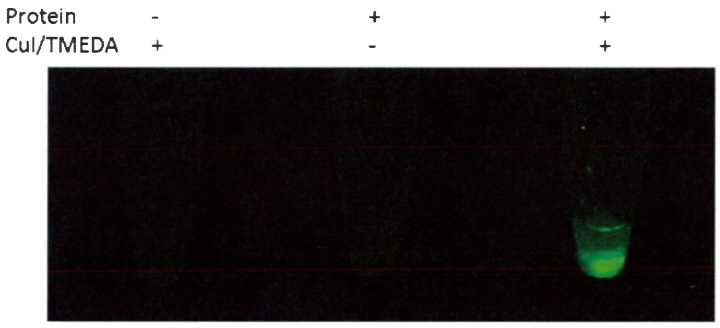


Figure 7.10: Bioconjugation of GFP-*p*PrF-151 with the hexynol derivatized Sephorose 6B resin. In the absence of either GFP or CuI/TMEDA catalyst, no fluorescence is observed; however, when all reaction components are present, successful immobilization of the GFP protein to the resin solid support is observed.

Reactions were then analyzed using a fluorescence detection multi-well plate reader and plotted to determine the relative fluorescence intensity of the control reaction versus the successful resin/protein coupling reactions. (Figure 7.11) It can be seen that the RFU of the Glaser-Hay reactions are about 10x higher than the RFU of the controls, further indicating the success of the Glaser-Hay reaction conditions to couple protein and an immobilized resin.

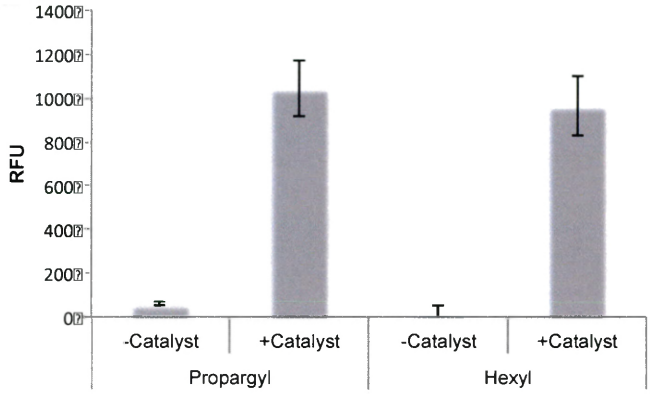


Figure 7.11: Fluorescence data of completed reactions with both propargyl alcohol and hexynol loaded Sepharose resins. Controls with no catalyst system indicate low background fluorescence attributed to only the resin, while mutant protein reacted with the catalyst and resin shows strong fluorescence. Standard deviations depicted in error bars.

III. Materials and Methods

General

Solvents and reagents, including the AlexaFluor 488 Alkyne, were obtained from either Sigma-Aldrich or Fisher Scientific and used without further purification. Epoxy-activated Sepharose 6B was obtained from GE Healthcare. Reactions were conducted under ambient atmosphere with solvents directly from the manufacturer. All GFP proteins were purified according to manufacturer's protocols using a Qiagen Ni-NTA Quik Spin Kit. Samples were analyzed on an Agilent 6520 Accurate-Mass Quadrupole-Time-of-Flight (Q-TOF) mass spectrometer equipped with an electrospray (ESI) ionization source and liquid chromatography (LC) (Agilent). Ionization settings were: positive mode; capillary voltage 3500 kV; fragmentor voltage 200 V; drying gas temperature 350 °C. Instrument was set

to standard 2 GHz, extended dynamic range and deconvolution was performed by Agilent MassHunter Qualitative Analysis software using the maximum entropy setting. To separate analyte a 2.1x150 mm, C8 reverse phase, wide pore (5 µm, 300 Å, Phenomenex) column was used with a water (A)/acetonitrile (B) (0.1% formic acid) gradient (2% B for 3 min, followed by a 2-95% B gradient over 15 min, and 95% B for 7 min).

Glaser-Hay Aqueous Conditions

Aqueous conditions for the Glaser-Hay reaction were optimized by preparing a phenylacetlyene homodimer. To a vial containing H_2O (3 mL) was added TMEDA (10 uL, 0.06 mmol) and CuI (10 mg, 0.05 mmol), forming the catalyst complex. Phenylacetylene (37 mg, 0.364 mmol) was then added and the reaction was allowed to stir at room temperature for 16 h. The reaction was extracted using EtOAc and H_2O washes (4 x 5 mL ea.), concentrated and dried *in vacuo*. The product was obtained as a white solid : 88 mg, 0.349mmol, 96% yield; ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.38-7.56 (m, J = 7.2 Hz, 10H).

Expression of GFP-*p*PrF-151

Escherichia coli BL21(DE3) cells were co-transformed with a pET-GFP-TAG-151 plasmid (0.5 µL) and pEVOL-*p*PrF plasmid (0.5 µL) using an Eppendorf electroporator. Cells were then plated on LB-agar plates supplemented with ampicillin (50 mg/mL) and chloramphenicol (34 mg/mL) and grown at 37 °C. After 16 h, single a single colony was selected and used to inoculate LB media (4 mL) supplemented with ampicillin and chloramphenicol. The culture was grown at 37 °C for 12 h. The culture was used to begin an expression culture of LB media (10 mL) at OD_{600} 0.1, then incubated at 37 °C, to an OD_{600} of ~0.6, at which point cells were induced with 1 M IPTG (10 uL), 20% arabinose (10 µL) and 100 mM *p*PrF (100 µL). Cultures were grown for an additional 16 h at 37 °C, then harvested by centrifugation (10 min at 10,000 rpm). The media was decanted and the cell pellet placed in the -80 °C freezer for at least 20 min. Purification was accomplished using

commercially available Ni-NTA spin columns and according to manufacturer's protocol. Protein yield and purity was assessed by SDS-PAGE, LC/MS, and spectrophotometrically using a Nanodrop spectrophotometer.

Protein-Fluorophore Glaser Hay Bioconjugation

The expressed GFP-*p*PrF-151 was coupled to AlexaFluor 488 alkyne using Glaser-Hay reaction conditions. In an eppendorf tube, 500 mM CuI (5 µL) and 500 mM TMEDA (5 µL) were mixed and equilibrated at 37 °C. After 10 minutes, 100 mM AlexaFluor 488 alkyne (10 µL) was added and equilibrated at 37 °C for 10 min. Finally, GFP-pPrF-151 (20 µL, 0.5 mg/mL) was added. A control reaction was also prepared with the same concentrations of fluorophore and protein, but with the catalyst system replaced with PBS buffer (10 µL). The reactions were incubated for various times at 37 °C. Reactions were then purified through centrifugal concentration on Spin-X UF colums (Corning), with wash cycles of PBS buffer (5 x 100 µL) until flow-through was free of fluorophore. The protein was then analyzed by SDS-PAGE gel to verify coupling of the fluorophore to the protein. Timecourse experiments were analyzed by comparing densiometry of fluorescent bands to their Coomasie Blue stained bands using a Biorad Molecular Imager Gel Doc XR+ system.

Immobilization of Alkynes onto Sepharose Resin

Epoxy-activated 6B Sepharose (GE Healthcare, 200 mg) was added to a filter syringe and washed with dH_2O (5 x 3 mL). Alkyn-ol (700 µmol) and coupling buffer (3.5 mL, pH 13.0) were added to a 15 mL falcon tube followed by the resin. The mixture was incubated at room temperature for 16 h. The resin was then transferred to a filter syringe and washed with coupling buffer (5 x 4 mL), and transferred to a 15 mL falcon tube with ethanolamine (3.5 mL). The resin was incubated at 30 °C for 4 h then washed in a filter syringe with 10 mM acetate buffer (pH 4) and Tris-HCl buffer (pH 8) for 3 cycles (4 mL ea.)

Attaching Immobilized resin to Protein

The expressed GFP-alkyne was then coupled to the immobilized resin using the Glaser-Hay reaction conditions. To eppendorf tube, 5 μL CuI (500 mM) and 5 μL TMEDA (500 mM) were combined and equilibrated at 37 °C for 10 minutes. To the Eppendorf was added 30 mg of immobilized resin and again equilibrated at 37°C. After an additional 10 minutes, 20 μL of concentrated GFP-alkyne was added. A control reaction was also set up using 30 mg immobilized resin, 20 μL GFP-alkyne, and 10 μL PBS buffer. The reactions were allowed to shake at 37 °C for six hours, 170 rpm. After six hours, reactions were washed on filter columns using PBS buffer (200 μL aliquots x 10). The washed resin-protein mixture was transferred to an Eppendorf tube and imaged for fluorescence.

Resin-Fluorophore Glaser Hay Conjugation

The alkyne derivatized resin was reacted under previously described conditions with the AlexaFluor 488. To an eppendorf tube, 500 mM CuI (5 μL) and 500 mM TMEDA (5 μL) were mixed and equilibrated at 37 °C for 10 min. Alkyne derivatized Sepharose (30 mg) was added and equilibrated at 37 °C. After an additional 10 minutes, 100 mM AlexaFluor 488 alkyne (5 μL) was added. A control reaction was also performed using 30 mg immobilized resin, AlexaFluor 488 alkyne (5 μL), and 10 μL 100 mM PBS buffer. The reactions were allowed to incubate at 37 °C for 6 h. The reactions were then washed on filter columns using 100 mM PBS buffer (10 x 200 μL). The fluorophore derivatized resin was transferred to an eppendorf tube and imaged for fluorescence using a BioRad Molecular Imager Gel Doc system.

Protein-Resin Glaser Hay Immobilization

The alkyne derivatized resin was reacted under previously described conditions with the GFP-*p*PrF-151 protein. To an eppendorf tube, 500 mM CuI (5 μL) and 500 mM TMEDA (5 μL) were mixed and equilibrated at 37 °C for 10 min. Alkyne derivatized Sepharose (30

mg) was added and equilibrated at 37 °C. After an additional 10 minutes, GFP-*p*PrF-151 (5 μL, 0.5 mg/mL) was added. . A control reaction was also performed using 30 mg immobilized resin, GFP (5 μL), and 10 μL 100 mM PBS buffer. The reactions were allowed to incubate at 37 °C for 6 h. The reactions were then washed on filter columns using 100 mM PBS buffer (10 x 200 μL). The protein immobilized resin was transferred to an eppendorf tube and imaged for fluorescence using a BioRad Molecular Imager Gel Doc system.

Acknowledgments

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Chapter 8-Natural Product Synthesis

I. Introduction

As described in Chapter 4, polyynes, or molecules containing an acetylenic scaffold, are known core structures in various natural products.[1-3] Over 1,000 of these naturally occurring molecules have been isolated from organisms such as plants, fungi, and coral.[2] These structures exhibit numerous biological activities including antibacterial, antifungal, anti-HIV, and anticancer properties.[3, 4] Therefore, synthetic routes to the preparation of these structures are necessary to further study their benefits and develop novel therapeutic analogs. Herein, we describe the utilization of our previously developed solid-supported Glaser-Hay methodology in order to synthesize some of these key polyyne natural products.

II. Results and Discussion

A. Using Solid-Supported Glaser-Hay couplings for Natural Product Synthesis

i. Toward Synthesis of 2,4-dodecadiynyl alcohol

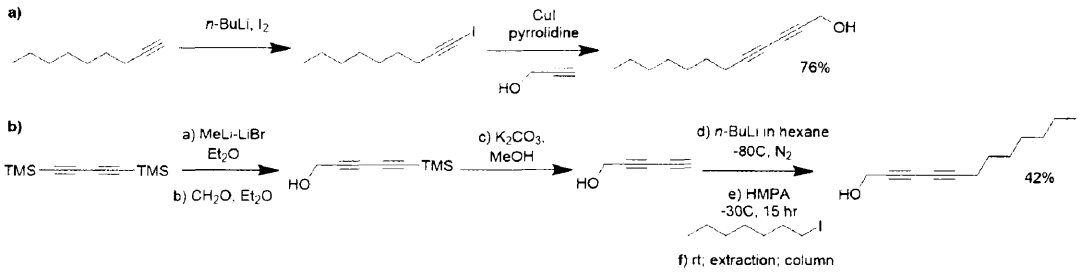
Many corals have been found to be rich in natural products that contain antibacterial, antifungal and cytotoxic properties. Specifically, numerous acetylenic polyynes have been isolated from the genus *Montipora*, a velvet coral.[5] One common product isolated is a diyne with a 7-carbon alkyl chain on one end and an alcohol on the other (**71**) (Figure 8.1).

71

Figure 8.1: 2,4-dodecadiynyl alcohol, a natural product isolated from *Montipora* is known to possess antibacterial properties, and has also been shown to exhibit cytotoxicity against human tumor lines.[2]

Numerous research groups have described the synthesis of this compound .[6,7,9,10] For example, Stefani et al. report this synthesis as seen in scheme 8.1a, with a reported yield of 76%.[10] However, this reported synthesis requires hazardous reagents, and requires a precursor step to synthesize the halogenated compound. Another reported synthesis by Fiandanese et al. can be seen in scheme 8.1b, and requires numerous and sometimes harsh reagents, six synthetic steps, and only results in a 42% yield.[9] This synthesis also required tedious purification steps post-reaction.

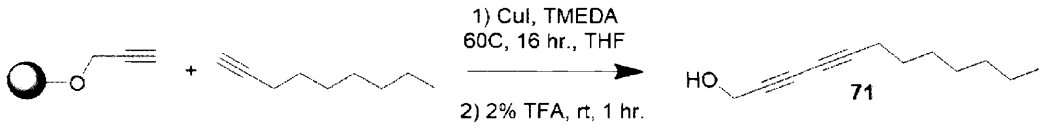
Scheme 8.1



We recognized that this polyyne is an asymmetric diyne and could be synthesized using the previously developed solid-supported Glaser-Hay coupling methodology.[8] In doing so, this asymmetric natural product could be synthesized using mild reaction conditions. By using a solid-support, a propargyl alcohol derivatized resin could be reacted

under Glaser-Hay conditions with a nine carbon terminal alkyne (1-nonyne) to afford **71** (Scheme 8.2).

Scheme 8.2

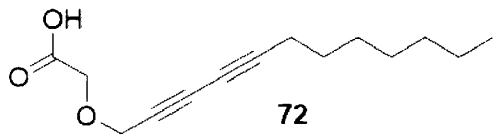


Using a trityl chloride resin with immobilized propargyl alcohol and a copper Iodide/TMEDA catalyst complex, a carbon bond is formed between 1-nonyne and propargyl alcohol. This initial reaction afforded the desired asymmetrical diyne still attached to the resin bead. Upon cleavage with trifluoroacetic acid in step 2, the desired asymmetrical natural product is obtained. Gratifyingly, upon initial reaction attempts, the desired product was obtained in 75% yield and in essentially a single synthetic step. Upon reaction completion, the product was purified through a quick silica plug. It was then analyzed via ^{1}H NMR and ^{13}C NMR. Spectral data was in accordance with previously reported literature values.[9] Overall, utilizing the solid-supported Glaser-Hay methodology to synthesize this product eliminated harsh reagents, halogenated precursor compounds, and synthetic steps required in previously reported syntheses, and produced the product in a better or comparable yield.

ii. Toward Synthesis of Montiporic Acid A

As noted, the coral genus *Montipora* possesses numerous biologically active polyyne natural products. A derivative of **71** described above, Montiporic Acid A (**72**) is

another common isolated polyyne from this velvet coral species (Figure 8.2). [2] Formerly, Montiporic Acid A has been isolated from the eggs of this coral and was shown to possess significant cytotoxicity against P-338 murine leukemia cells. It has also been proven to be an efficient antibacterial agent against *E. coli*.[9]



72

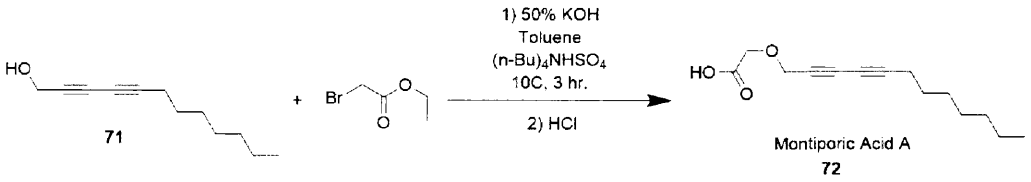
Figure 8.2: Montiporic Acid A, typically isolated from *Montipora* and known to possess antibacterial properties toward *E. coli* and also shown to exhibit cytotoxicity against P-338 murine leukemia cells.

A successful synthetic approach of Montiporic Acid A was developed by Stefani et al. in 1999. In their synthesis, this research group converted 1-nonyne to 1-iodo-l-nonyne by treatment with *n*-BuLi and Iodine. This product was then coupled with propargyl alcohol in the presence of pyrrolidine/CuI to produce dodeca-2,4-diynol (**71**). (Scheme 8.1a) The synthesis concluded with the reaction of this product with ethyl 2-bromoacetate and an $(n\text{-}Bu)_4NHSO_4$/KOH complex to afford Montiporic Acid A. The total synthesis reported by Stefani et. al. of Montiporic Acid A resulted in a 56% yield.[10] Fiandese et. al. report the same approach to the desired montiporic acid A using **71** as a starting material and are able to obtain the desired product in a 34% net yield. Overall, what varied was these two groups' synthetic routes to the desired polyyne starting material (**71**) (See scheme 8.1). As described, both of these synthetic schemes required

numerous synthetic steps and harsh reagents. We envisioned that by using our previously developed solid-supported Glaser-Hay methodology to synthesize the precursor, **71**, with mild reaction conditions, we would provide a means to synthesize the desired Montiporic Acid A in fewer reaction steps, with mild reaction conditions, and higher purity due to the use of the solid support.

As noted, our approach using the solid-supported Glaser-Hay reaction methodology was successful for **71**. Therefore, efforts moved forward in order to use this synthesized natural product to obtain Montiporic Acid A. A reaction protocol from the literature[9] was followed, adding ethyl 2-bromoacetate to the polyyne precursor and using tetrabutylammonium bisulfate as a phase transfer catalyst. (Scheme 8.3)

Scheme 8.3



Following the reaction, an extraction was performed using consecutive washes of DCM and H_2O. The product was then analyzed via ^{1}H and ^{13}C NMR. Results indicated that by using our previously synthesized **71** precursor for the reaction, the desired product was formed in a net yield of 49%. Analysis shows product in moderate to good purity but future work is required to further purify the desired Montiporic Acid A and increase the synthetic yield.

iii. Toward Synthesis of Octatriyn-1-ol

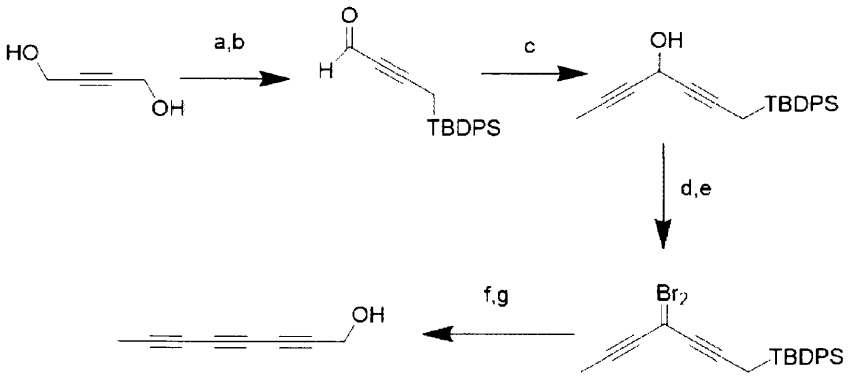
Fungi can also be a rich source of polyyne natural products. Octatriyn-1-ol, pictured in figure 8.3 (**73**), was originally isolated by Hearn and coworkers from an edible mushroom, *Kuehneromyces mutabilis*.[11] This molecule, as well as similar structures were shown to possess antibacterial activities.

73

Figure 8.3: Octatriyn-1-ol, formerly isolated from fungi and shown to have antibacterial activity.

This product has been synthesized successfully by means of a Fritsch Buttenberg-Wiechell rearrangement by Luu et al.[12] The protocol for the generation of the triyne core performed by this group can be seen in scheme 8.4.

Scheme 8.4

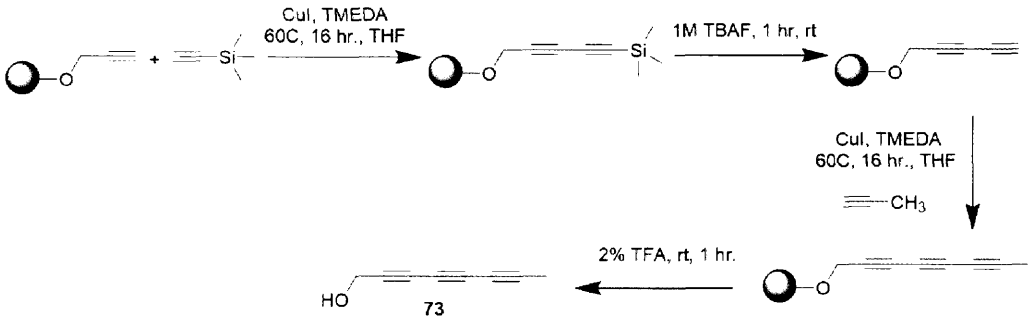


a)TBDPSCl, THF, Et_3N, DMAP; b) $BaMnO_4$; c) CH_3CCLi, LiBr, THF, -78 °C; d) $BaMnO_4$; e) CBr_4, PPh_3, CH_2Cl_2, Et_3N, 0 °C; f) BuLi, hexanes, -78 °C; g) TBAF, THF, -20 °C.[12]

Overall, this previously reported synthesis requires eight synthetic steps to generate the compound. This reported methodology also requires numerous reagents-some hazardous, and careful reaction temperature control leading to an overall yield of only 3%.[12]

Building on our reported methodology and protocol for extending the acetylenic scaffold,[8,13] here we report a synthetic route toward Octatriyn-1-ol (Scheme 8.5), decreasing the synthetic steps and number of reagents used from previous reports as well as increasing yield. Beginning with a propargyl alcohol immobilized resin, a TMS group was added using the Glaser-Hay reaction. The TMS group was then cleaved using a TBAF solution, regenerating the terminal alkyne. Propyne was then coupled to the resin using the Glaser-Hay reaction and the product was cleaved with a 2% TFA solution, affording the desired asymmetrical triyne natural product, **73**.

Scheme 8.5



Utilizing our described methodology, and employing a silica plug using 5:1 hexanes:EtOAc for purification, we have been successful at the synthesis of **73** (characterization data may be found in Materials and Methods). This compound was acquired in good purity and with a yield of 68%.

iv. Toward Synthesis of Agrocybin

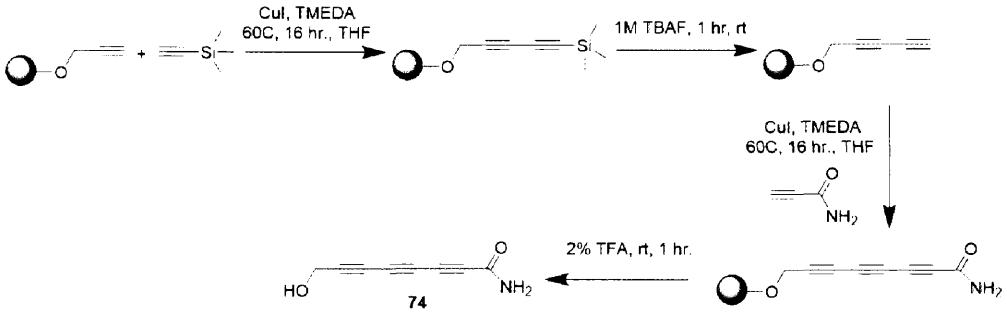
Another natural product found in fungi is agrocybin, originally isolated from *Agrocybe dura*.[2] Naturally, this molecule is released by *Agrocybe* dura during the fruiting season as an antifungal agent. Furthermore, it has been shown to exhibit antibacterial properties. This product has a triyne core and an amide capping group. (Figure 8.4)

74

Figure 8.4: The structure of Agrocybin, originally found in the fungi species *Agorocybe dura* and known to possess important antifungal and antibacterial properties.

A similar approach used to access **73** was attempted to synthesize agrocybin. Based on a previously established protocol,[8,13] a known methodology is available for preparation of the alcohol and the extended polyyne core. We then envision adding the desired cap, propiolamide, in order to generate the product via the Glaser-Hay reaction. Upon reaction completion, the cleaved material required quick purification via a short silica plug (Scheme 8.6) yielding **74**.

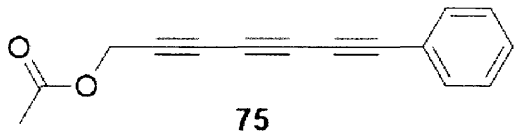
Scheme 8.6



A Glaser coupling approach to agrocybin synthesis was successfully performed by Jente and co-workers in 1984 to allow NMR studies on this molecule and its derivatives.[14] . To synthesize the agrocybin, this research group employed Glaser reaction conditions with an extended propargyl alcohol and propiolamide. Because of the chemoselectivity limitations associated with the Glaser protocol (See Chapter 4), further purification was necessary and yields were reported as only 30%.[14] We hope that utilization of our novel solid-supported Glaser-Hay methodology will drastically increase the yield of this product and provide a direct route to its synthesis. Unfortunately, initial attempts to obtain this product using our methodology (Scheme 8.4) did not indicate the presence of product upon NMR analysis. Future work needs to be employed, including potentially protecting the propiolamide prior to coupling as well as scaling up the reaction and completely purifying the completed product for accurate analysis.

v. Toward Synthesis of phenylhepta-2,4,6-triynyl acetate

The last natural product we attempted to synthesize in order to demonstrate the utility of the solid-supported Glaser-Hay methodology was **75**. This acetate polyyne has been isolated from several species of *Bidens* in the Aster family of plants and has shown antibacterial properties.[2] (Figure 8.5)

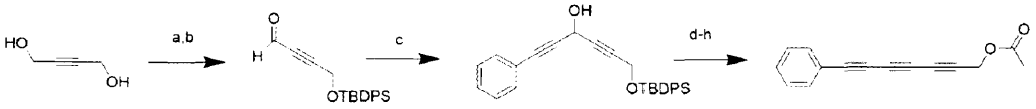


75

Figure 8.5: Natural product phenylhepta-2,4,6-triynyl acetate consisting of a triyne core asymmetrically capped with a phenyl group and an acetate group. This product has been previously isolated from many plants species including Bidens.[2]

A previously reported synthesis uses a starting material of 1,4 butynediol and TBDPS as a protecting group in order to react with a lithiated phenylacetylene and undergo further cycles of Fritch-Butenberg-Wiechell rearrangements to produce the triyne core.[12] (Scheme 8.7)

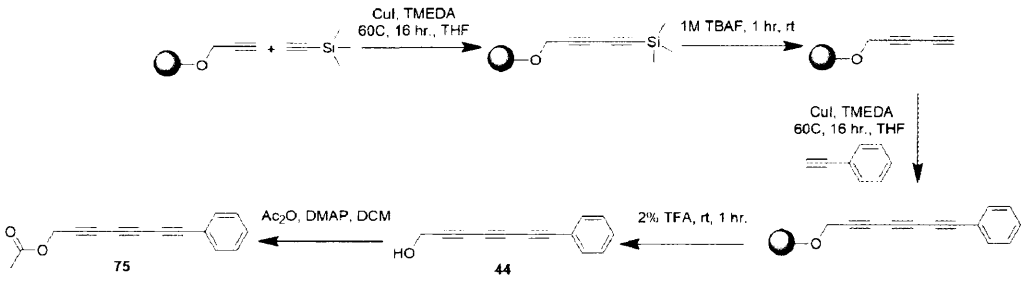
Scheme 8.7



a) TBDPSCl, DMAP, THF; b) $BaMnO_4$, CH_2Cl_2; c) PhCCLi, THF, -78°C; d) $BaMnO_4$; CH_2Cl_2, rt e) CBr_4, PPh_3, CH_2Cl_2, Et_3N, 0 °C; f) BuLi, hexanes, -78 °C; g) TBAF, THF, rt; h) Ac_2O, DMAP, NEt_3, CH_2Cl_2, rt.

This methodology is similar to that shown in Scheme 8.4 performed by the same research group, and totals eight synthetic steps to achieve the desired product. It also requires numerous reagents, sometimes harsh, and only affords the product in a 14% yield.[12] Here we propose utilizing our previously reported molecule **44** [13] and envision simply acetylating the alcohol to yield **75**. (Scheme 8.8) Overall, our methodology employs mild reaction conditions and eliminates synthetic steps from the previously reported synthesis.

Scheme 8.8



The previously described Glaser-Hay coupling with TMS followed by TBAF induced TMS deprotection was employed to prepare the immobilized polyyne core, and the polyyne was capped with phenylacetylene. The solid-support was then cleaved to afford the triynol product. The alcohol was acetylated with acetic anhydride in the presence of DMAP to afford the desired product. Upon acetylation, the reaction was extracted and washed with DCM/H_2O and dried with $MgSO_4$. The triyne **75** was then purified on a silica column with 5:1 Hex/EtOAc. This synthesis afforded the desired natural product, which

was then analyzed via NMR. (9 mg, 0.041 mmol, 46%) Utilizing the solid supported Glaser-Hay methodology we were able to eliminate synthetic steps as well as harsh and excessive reagents, and drastically improve the yield of this natural product.

Overall, we have shown that solid-supported Glaser Hay reactions are a useful methodology in the synthesis of natural products. Due to possible purification issues throughout the reactions, and small reaction scales, more research is required in order to obtain pure products and hopefully increase the yields provided by the methodology. Future work will employ examining these products for biological activity in an anti-bacterial screen in order to potentially elucidate new therapeutic agents.

III. Materials and Methods

General. Solvents and reagents were obtained from either Sigma-Aldrich or Fisher Scientific and used without further purification, unless noted. Tritylchloride resin, 100-200 mesh, 1% DVB crosslinking, was purchased from Advanced Chemtech. Reactions were conducted under ambient atmosphere with non-distilled solvents. NMR data was acquired on a Varian Gemini 400 MHz. GC/MS analysis was conducted on an Agilent Technologies 6890N GC system interfaced with a 5973N mass selective detector. An Agilent J&W GC capillary column (30 m length, 0.32 mm diameter, 0.25 nm film) was employed with a splitless injection (250 ˚C inlet, 8.8 psi) with an initial 70 ˚C hold (2 min) and ramped for 15 min to 230 ˚C.

Immobilization of Alcohol onto Trityl Chloride Resin in Low Loading Conditions

Trityl chloride resin (200 mg, 0.36 mmol, 1 equiv.) was added to a flame dried vial charged with dichloromethane (5 mL). The resin was swelled at room temperature with gentle

stirring for 15 min. Alcohol (25.0 μL, ~1.2 equiv.) was added to reaction, followed by triethylamine (10.0 μL, 0.072 mmol, 0.2 equiv). The mixture was stirred at room temperature for 16 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each). The resin was swelled in CH_2Cl_2 and dried under vacuum for 45 min before further use.

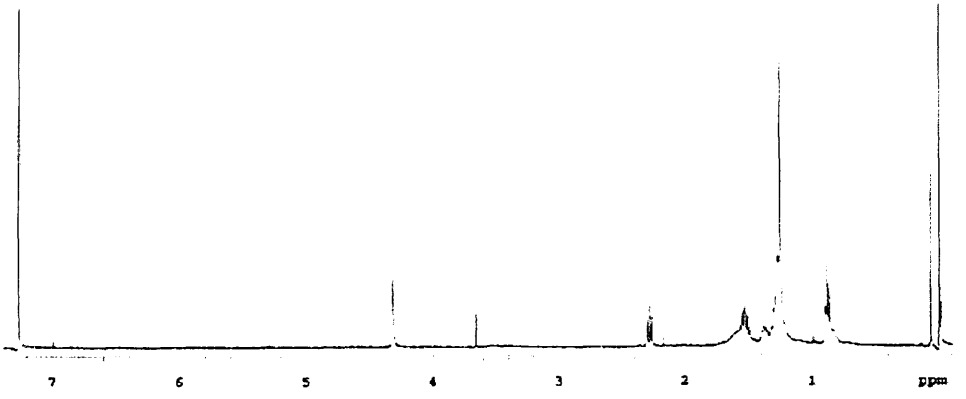
Polyyne Extension Protocol

Trimethylsilylacetylene (160 μL, 1.05 mmol, 15 equiv.) was added to a flame dried vial containing the alcohol derivatized trityl resin (100 mg, 0.07 mmol, 1 equiv.), and tetrahydrofuran (2.0 mL). The CuI (20 mg, 1.06 mmol) and tetramethylethylenediamine (20 μL, 0.132 mmol.) were added to a separate flame-dried vial then dissolved in tetrahydrofuran (2.0 mL). The catalyst mixture was then added to the resin in one portion and stirred at 60 ˚C for 16 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each). The TMS group was then cleaved by incubation in 1M tetra-n-butylammonium fluoride trihydrate in DCM (TBAF, 1 mL, 1 h). Then the reaction was again transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each) and dried under vacuum for 45 minutes. Product was weighed and transferred to flame dried vial for future use.

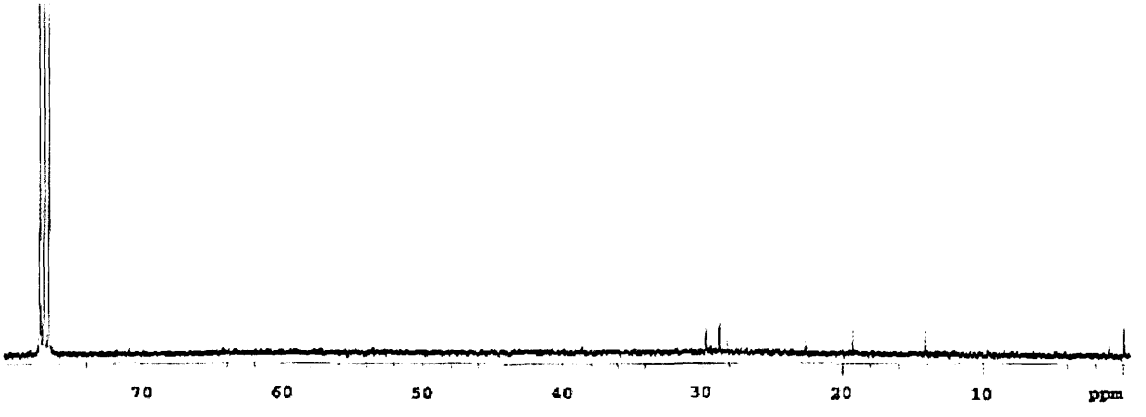
71. 2,4-dodecadiynyl alcohol. 1-Nonyne (115 μL, 0.70 mmol, 10 equiv.) was added to a flame dried vial containing the propargyl alcohol derivatized trityl resin (100 mg, 0.070 mmol, 1 equiv.), and tetrahydrofuran (2 mL). The copper catalyst (10 mg, 0.053 mmol, ~0.7 equiv.) and tetramethylethylenediamine (30 μL) were added to a separate flame-dried vial then dissolved in tetrahydrofuran (2 mL). The catalyst mixture was then added to the resin in one portion and stirred at 60 ˚C for 16 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each). The product was then cleaved from the resin by treatment with 1 mL 2% TFA (DCM, 1 h), and filtered into a vial. A short silica plug was utilized to remove unreacted starting material (1:1 EtOAc/Hex) and pure product was obtained (0.010 g, 0.052 mmol, 75%). ^{1}H NMR:

400 MHz, $CDCl_3$: δ = 4.27 (s, 2H), 2.24 (t, *J* = 7.2 Hz, 2H), 1.55 (quint, *J* = 7.2 Hz, 2H), 1.39–1.31 (m, 2H), 1.30–1.19 (m, 6H), 0.90 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR of Polyyne 1, 400 MHz, $CDCl_3$: δ = 51.7, 31.8, 29.5, 28.7, 28.1, 22.4, 19.2, 14.0.

^{1}H NMR **71:**



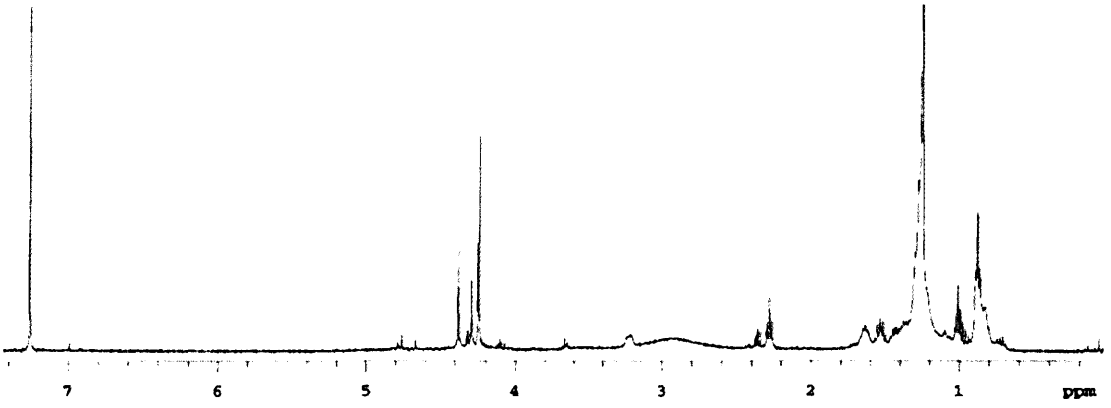
^{13}C NMR **71:**



72. Montiporic Acid A. Ethyl bromoacetate (10 µL, 0.1 mmol, 2 equiv.) was added at 10 °C to a vial containing **71** (0.010 g, 0.052 mmol, 1 equiv.) dissolved in toluene (1 mL), 50% KOH (200 µL), and tetra-butyl ammonium sulfate (10 mg, 0.03 mmol, ~0.5 equiv.). The reaction was then vigorously stirred at 10 °C for 3 hours. Upon completion, the reaction

was quenched with dilute HCl (5 mL), extracted with EtOAc, and washed with H_2O. (3x5 mL) The product was then dried over anhydrous $MgSO_4$ and solvent removed *in vacuo*. Purification was performed via column chromatography (hexanes: EtOAc 10:1 to 1:3) to yield the desired product (8 mg, 0.039 mmol, 49%). ^{1}H NMR: 400 MHz, $CDCl_3$: δ = 4.38 (s, 2H), 4.21 (s, 2H), 2.24 (t, J = 7.2 Hz, 2H), 1.49 (quint, J = 7.2 Hz, 2H), 1.34–1.31 (m, 2H), 1.29–1.19 (m, 6H), 0.84 (t, J = 6.9 Hz, 3H)

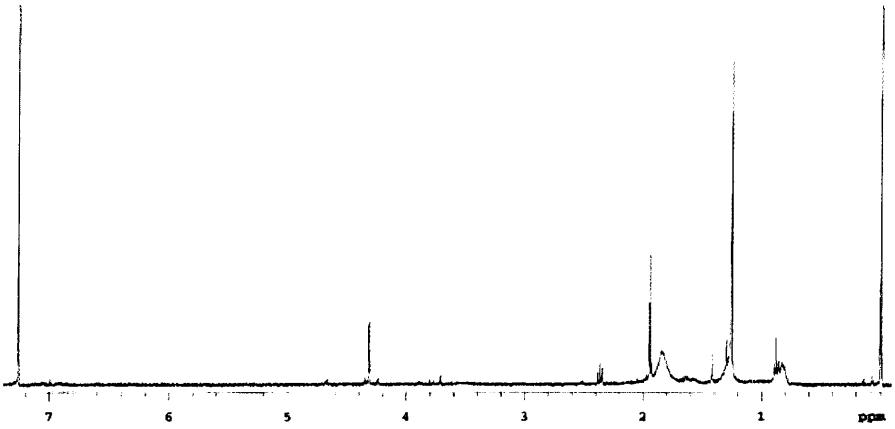
^{1}H NMR **72:**



73. Octatriyn-1-ol. The previously described polyyne extension protocol was used to obtain the immobilized terminal alkyne diyne. 1-Propyne (53 µL, 0.70 mmol, 10 equiv.) was added to a flame dried vial containing the immobilized resin (100 mg, 0.07 mmol, 1 equiv.) and tetrahydrofuran (2 mL). The copper catalyst (10 mg, 0.053 mmol, ~0.7 equiv.) and tetramethylethylenediamine (30 µL) were added to a separate flame-dried vial then dissolved in tetrahydrofuran (2 mL). The catalyst mixture was then added to the resin reaction in one portion and stirred at 60 °C for 16 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 3 mL each). The product was then cleaved from the resin by treatment with 1 mL 2% TFA (DCM, 1 h), and filtered

into a vial. A short silica plug was performed to remove unreacted starting material (5:1 EtOAc/Hex), affording product **73.** (0.004 g, 68%) ^{1}H NMR of Triynol: 400 MHz, $CDCl_3$: δ = 4.70 (s, br, 1H), δ = 4.34 (s, 2H), 1.96 (s, 3H)

^{1}H NMR **73:**

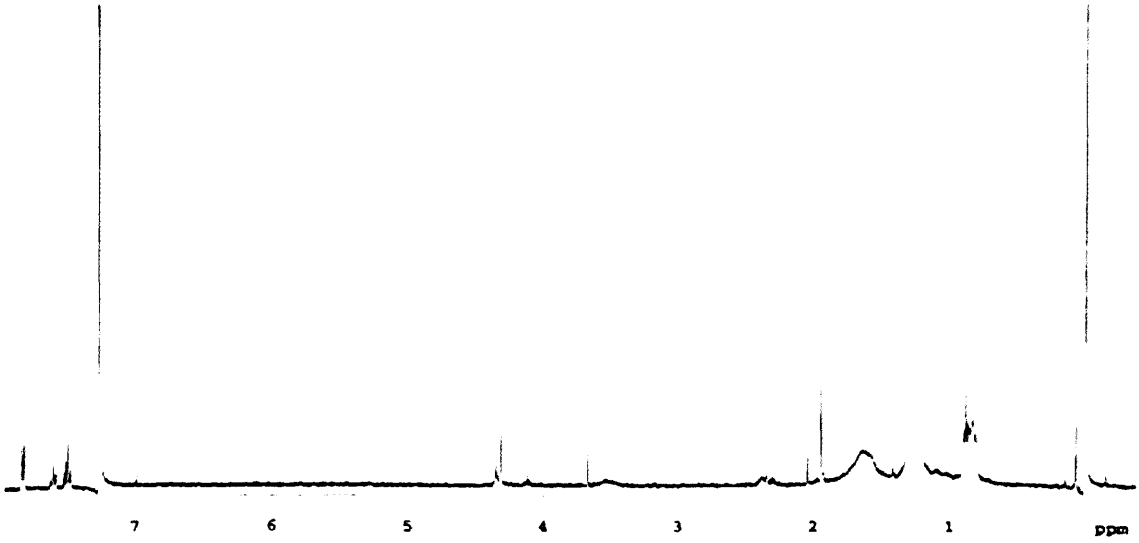


74. Agrocybin. The previously described polyyne extension protocol was used to obtain the immobilized terminal alkyne diyne. Propiolamide (10 equiv.) was added to a flame dried vial containing the starting material (100mg) and tetrahydrofuran (4 mL). The copper catalyst (10 mg, 0.053 mmol) and tetramethylethylenediamine (30 µL) were added to a separate flame-dried vial then dissolved in tetrahydrofuran (1 mL). The catalyst mixture was then added to the resin reaction in one portion and stirred at 60 °C for 16 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 3 mL each). The product was then cleaved from the resin by treatment with 1 mL 2% TFA (DCM, 1 h), and filtered into a vial. A short silica plug was attempted to remove unreacted starting material (EtOAc/Hex).

75. phenylhepta-2,4,6-triynyl acetate. The previously described polyyne extension protocol was used to obtain the immobilized terminal alkyne diyne. Phenylacetlyene

(0.70 mmol, 10 equiv.) was added to a flame dried vial containing the starting material (100 mg, 0.070 mmol, 1 equiv.) and tetrahydrofuran (2 mL). The CuI (10 mg, 0.053 mmol) and tetramethylethylenediamine (30 μL) were added to a separate flame-dried vial then dissolved in tetrahydrofuran (2 mL). The catalyst mixture was then added to the resin reaction in one portion and stirred at 60 °C for 16 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (5 alternating rinses with 5 mL each). The product was then cleaved from the resin by treatment with 1 mL 2% TFA (DCM, 1 h), and filtered into a vial. Solvent was removed *in vacuo* to afford the 7-phenyl-trynol (10 mg, 0.056 mmol, 80%) Acetic anhydride (1 mL) and a catalytic amount of DMAP were added and dissolved in 1 mL DCM. The reaction was allowed to stir at room temperature for 3 hr, followed by an extraction using DCM/H_2O (3x5 mL) and drying with MgSO4. The product was then purified on a silica gel column using 5:1 hex:EtOAc yielding **75** (9 mg, 0.041 mmol, 46%), which was then analyzed via ^{1}H NMR: 400 MHz, $CDCl_3$: δ = 7.40-7.25 (m, 5H), 4.35 (s, 2H), 1.96 (s, 3H).

^{1}H NMR 75:



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